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ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS JOURNAL

SEPTEMBER/OCTOBER 1984
VOL. 67, NO. 5

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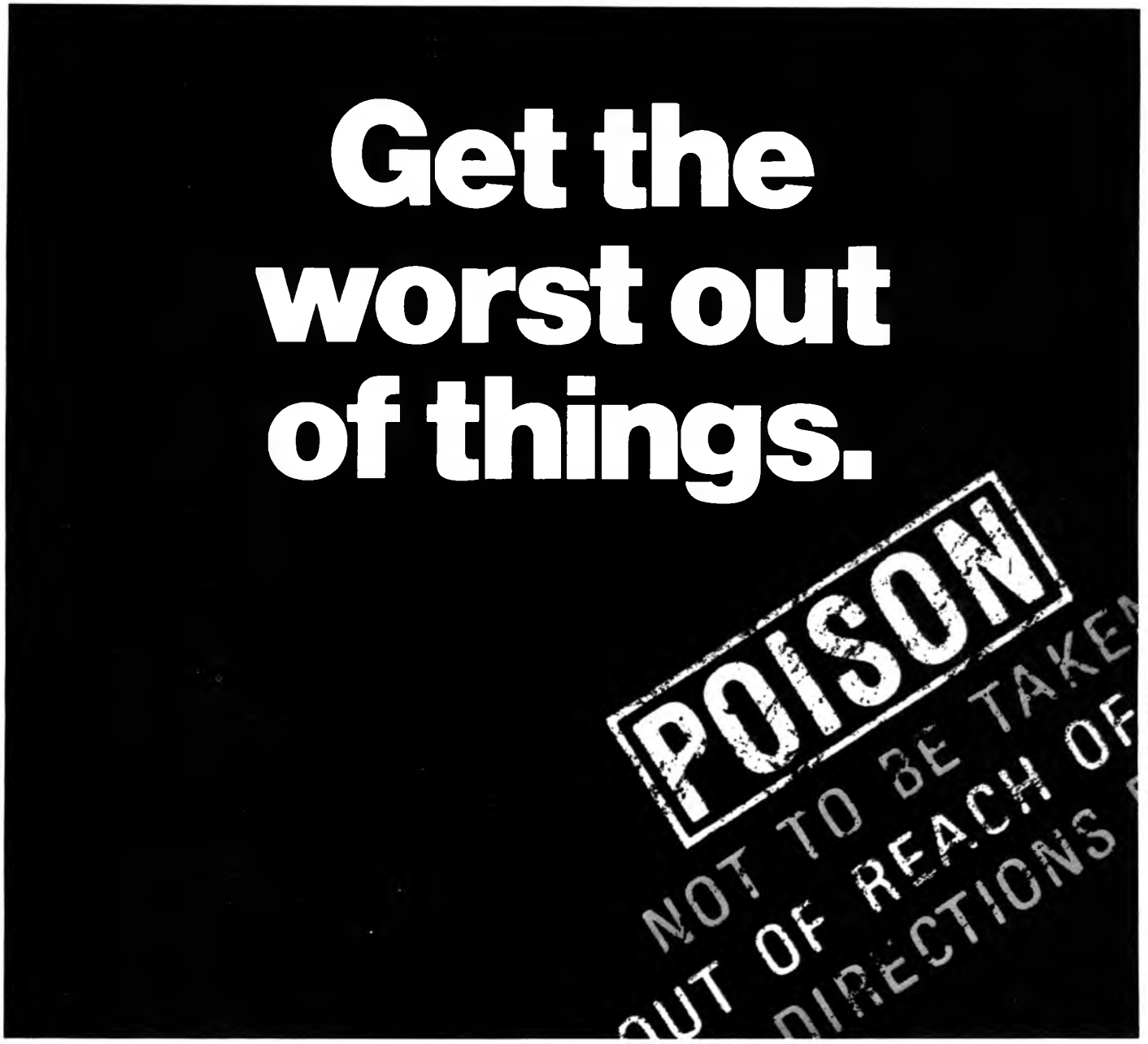

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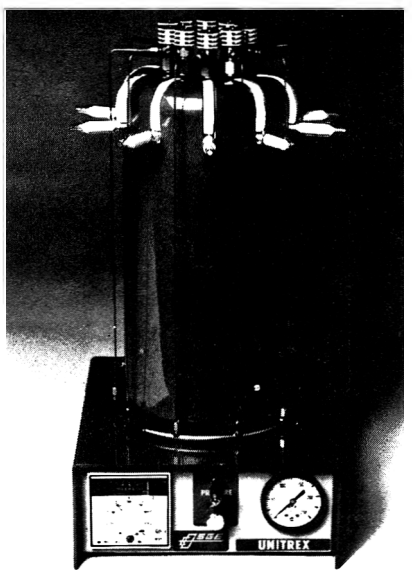

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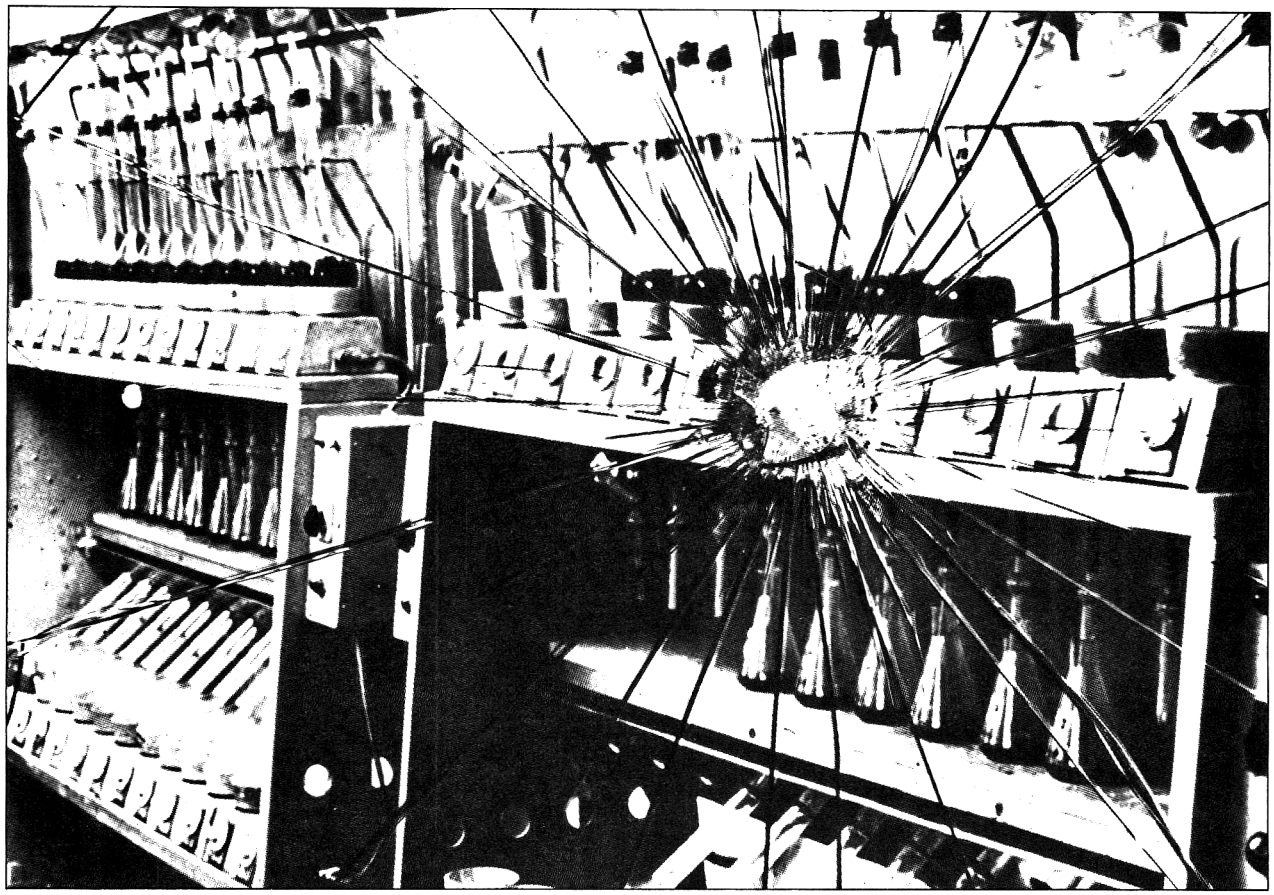

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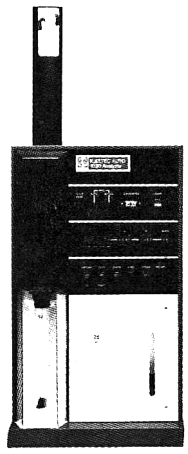

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ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS JOURNAL

Basic & Applied Research in the Analytical Sciences Related to Agriculture and the Public Health

SEPTEMBER/OCTOBER 1984
VOL. 67, NO. 5

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Circle No. 353

Variable Speed Metering Pump

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Circle No. 355

Gamma Counter

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Circle No. 356

Headspace GC

The HS-100 automatic headspace sampler, developed for the Sigma 2000 GC, uses pneumatic injection and a microprocessor system that loads samples from the 100-sample storage magazine to the thermostatted carousel. The HS-100 performs multiple headspace extractions, may be operated in the high pressure mode, and comes with a wide variety of septa for different applications. Contact: Perkin-Elmer Corp., Main Ave, Norwalk, CT 06856; 203/762-1000. Telex: 965-954.
Circle No. 357

Database

The Wiley/NBS Mass Spectral Database combines information from John Wiley & Sons' Registry of Mass Spectral Data and the National Bureau of Standards' National Institute of Health-Environmental Protection Agency (NIH-EPA) Database. It offers direct access to more than 80 000 spectra of about 68 000 different chemical compounds. The database is available through Hewlett-Packard for its HP 5987A GC/MS System and is on magnetic tape from Wiley. Contact: Gary Craig, John Wiley & Sons, 605 Third Ave, New York, NY 10158; 212/850-6360.
Circle No. 358

Autozero Accessory

The Autozero Accessory Module will zero any detector output remotely by a switch closure from any data system controller or autosampler. The module compensates for ± 0.7 AU of background offset. Contact: Terry F. Meyers, LDC/Milton Roy, 10235 Riviera Beach, FL 33404; 305/844-5241 (Florida), 800/327-6182 (toll-free).
Circle No. 359

Gel Casting System

The UltroMould Gel Casting System for production of thin and ultra-thin gels for analytical electrophoresis and electrofocusing is easy to use, versatile, and bubble-free. Trapped air is easily removed by sliding the upper

plate backward to allow the bubble to escape. Continuous adjustment of the speed and direction of the sliding plate during filling guarantees perfect gels. Interchangeable spacer windows assure excellent reproducibility by providing accurate and uniform gel thicknesses. Contact: LKB Instruments, Inc., 9319 Gaither Rd, Gaithersburg, MD 20877; 301/963-3200.
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Bio-Rad's refrigerated automatic sampler deals with many problems associated with sample stability in LC analysis, such as enzymatic activity, chemical degradation, and sample evaporation. Model AS-48 performs up to 9 multiple analyses each of up to 48 samples with a pressure rating to 5000 psi and a sample size from 5 to 1500 μL. Sample temperature can be lower than 10°C at 25°C ambient and environment can be ambient from 10 to 35°C. Contact: Bio-Rad Chemical Division, 2200 Wright Ave, Richmond, CA 94804; 415/234-4130. Telex: 335-358.
Circle No. 361

Chemical Screening System

Mutascreen®, a fully automated, tabletop system, can be used to detect cancer-causing drugs, pesticides, and other chemicals. The system is based on the fact that carcinogens cause mutations, and testing is done on special bacteria that have lost the ability to manufacture histidine to determine if these bacteria are genetically altered by the chemical in question. Up to 50 samples can be analyzed in 24 h. Applications include new food, drug, agricultural, and industrial chemicals, industrial air and water emission, and industrial workplace. Contact: Casey P. Eitner, Labsystems Inc., PO Box 48723, Chicago, IL 60648; 312/967-5220.
Circle No. 362

LC Column

The Bio-Gel® TSK SP-5PW cation-exchange LC column contains a strong cation, polymeric resin which permits a pH 2–12 operating range for flexibility in protein separations and column cleanup. The column separates proteins up to 10^6 Daltons and has a capacity of 1–5 mg. Contact: Bio-Rad Laboratories, 2200 Wright Ave, Richmond, CA 94804; 415/234-4130. Telex: 335-358.
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The portable, piston sampling vessel provides a safe, reliable way to transport liquid or gas hydrocarbon samples. A sample can be removed without changing the pressure of the product or exposing it to contaminants, and the free-moving piston maintains sample consistency. Contact: Bob Parks, Big 3 Industries, PO Box 1026, LaPorte, TX 77571; 713/474-4477 (or 1-800/392-5841).
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LITERATURE

Gas Chromatographs

The MEGA Series and its modular components are described in a 12-page brochure. The microprocessor-based automation package includes an automatic liquid injector and headspace analyzer combined with an optional multifunction processor. The brochure also discusses the oven design with optimized column environment, a variety of injectors, detectors, sample preparation instruments, and capillary columns. Contact: Erba Instruments, Inc., 4 Doulton Pl, Peabody, MA 01960; 617/535-5986.
Circle No. 365

Scientific Apparatus and Process Equipment

The Tekmar® '84 catalog contains over 20 new items and features automatic titrators, an automatic headspace concentrator, a quality control viscometer, precision automatic pipette tips and microtubes, and glass-sealed pH buffers. Tekmar's areas of specialization are sample preparation, volatile organic analysis, eletrochemical analysis, viscosity/rheology, quality control viscometers, tissue culture, and process equipment. Contact: Tekmar Co., PO Box 371856, Cincinatti, OH 45222; 800/543-4461. (In Ohio and Canada, call collect 513/761-0633.) Telex: 21-4221.
Circle No. 366

Water Analysis

The 1984/85 Thomas Scientific Water Analysis Catalog contains over 2500 items with photographs and illustrations, and features LaMotte water testing kits, instruments, and associated apparatus. Also included is a listing of apparatus and reagents needed in water/wastewater testing from over 135 manufacturers. Contact: Thomas Scientific, Vine St at Third, PO Box 779, Philadelphia, PA 19105-0079; 215/574-4500.
Circle No. 367

Standard Reference Materials

The 1984/1985 Standard Reference Materials catalog contains about 90 new SRMs and 270 renewal SRMs, with an enclosed price list. For information about even newer SRMs not included in the catalog and about obtaining the catalog, contact: Office of Standard Reference Materials, Room B311, Chemistry Building, National Bureau of Standards, Washington, DC 20234; 301/921-2045
Circle No. 368

LC Products

The Shandon 1984 24-page guide for LC columns, packing accessories, and column packing materials includes specific applications for Shandon's spherical packing material, Hypersil, that are illustrated with chromatograms. Information on a column-packing pump is also included. Contact: Mary Ellen Goffredo, Shandon Southern Instruments, Inc., 515 Broad St, Drawer 43, Sewickley, PA 15143; 412/741-8400.
Circle No. 369

Glassware

The second edition of the Scientific Glassware Catalog has over 3000 pages listing 7000 items from macro to process glassware, used for distillation, reaction, and environmental work done in industrial, research, educational, government, and medical labs. Contact: Southeastern Laboratory Apparatus, 118 Murrah Rd, North Augusta, SC 29814; 803/279-7668.
Circle No. 370

Sialic Acid Determination

The UV-Vis application note (UV-21) covers sialic acid determination using the Varian DMS 90 UV-Vis Spectrophotometer. A table and a sialic acid assay graph are used to illustrate results obtained in the experiment. Contact: Jack Aeschliman, Varian Instrument Group, 220 Humboldt Ct, Sunnyvale, CA 94089; 408/734-5370.
Circle No. 371

BOOKS IN BRIEF

Topics in Forensic and Analytical Toxicology. Edited by R. A. A. Maes. Published by Elsevier Science Publishers, PO Box 211, 1000AE Amsterdam, The Netherlands, 1984. Also available from Elsevier Science Publishing Co., Inc., PO Box 1663 Grand Central Station, New York, NY 10163. 224 pp. Price: $57.75 U.S. & Canada/Dfl. 150.00. ISBN 0-444-42313-3.

Containing the invited and selected papers from the Annual European Meeting of the International Association of Forensic Toxicologists, this volume covers some of the following topics: drugs and driving; drugs of abuse; toxicological analysis of drugs, their metabolites and their clinical and forensic implications; mass spectrometry in toxicology; combination methodology in toxicological analysis; systematic toxicological analysis; and toxicology in developing countries.

Analytical Spectroscopy. Edited by W. S. Lyon. Published by Elsevier Science Publishers, PO Box 211, 1000AE Amsterdam, The Netherlands, 1984. Also available from Elsevier Science Publishing Co., Inc., PO Box 1663 Grand Central Station, New York, NY 10163. 408 pp. Price: $75.00 U.S. & Canada/Dfl. 195.00. ISBN 0-444-42312-5.

This volume contains the Proceedings of the 26th Conference on Analytical Chemistry in Energy Technology, with detailed discussions on laser spectroscopy and mass spectroscopy. Also appearing are articles on resonance ionization mass spectrometry; new and improved techniques and applications of plasma spectroscopy, including hot cell operations; and advances in nuclear techniques and gamma spectrometry.

Mycotoxins—Production, Isolation, Separation and Purification. Edited by V. Betina. Published by Elsevier Science Publishers, PO Box 211, 1000AE Amsterdam, The Netherlands, 1984. Also available from Elsevier Science Publishing Co., Inc., PO Box 1663 Grand Central Station, New York, NY 10163. 536 pp. Price: $113.50 U.S. & Canada/Dfl. 295.00. ISBN 0-444-42289-7.

Covering more than 250 mycotoxins, Part 1 discusses the occurrence, production, biological activities, and biochemical modes of action of mycotoxins, along with applications of chromatographic techniques. Part 2 discusses key chemical families, the most important individual mycotoxins, data on producing organisms, and the physical, chemical, and biological properties of mycotoxins, followed by methods of production, isolation, purification, chromatographic characterization, detection, and assays.

High-Performance Liquid Chromatography of Proteins and Peptides. Edited by M. T. W. Hearn, F. E. Regnier, and C. T. Wehr. Published by Academic Press, 111 Fifth Ave, New York, NY 10003, 1983. Also available from Academic Press, 24/28 Oval Rd, London NW1 7DX, England. 288 pp. Price: $27.50. ISBN 0-12-335780-2.

This book contains the Proceedings of the First International Symposium of HPLC of Proteins and Peptides, which covered size exclusion, ion-exchange, and reverse phase chromatography, as well as use of HPLC techniques in protein structural studies and peptide isolation, detector technology, and specialized separation methods.

The Peptides: Analysis, Synthesis, Biology. Volume 5: Special Methods in Peptide Synthesis. Edited by E. Gross and J. Meienhofer. Published by Academic Press, 111 Fifth Ave, New York, NY, 1983. Also available from Academic Press, 24/28 Oval Rd, London NW1 7DX, England. 536 pp. Price: $79.50/$69.50 (subscription price). ISBN 0-12-304205-4.

Topics covered include: recombinant DNA methods, acidolytic deprotection procedures in peptide synthesis, side reactions in peptide synthesis, quantitation and sequence dependence of racemization in peptide synthesis, α,β-dehydroamino acids and peptides, and unusual amino acids in peptide synthesis.

Analytical Methods for Graphite Tube Atomizers. Available from Varian Instrument Group, 220 Humboldt Ct, Sunnyvale, CA 94089, 1984. 132 pp. Price: $75.00. Part No. 85-100447-00.

This review of graphite furnace techniques includes the following methodologies: air particulate analysis, agricultural, biological and biochemical, geochemical and metallurgical, petroleum, and water analysis. How to enhance analytical performance and effects of interference and background absorption are also discussed.

Drug Resistance in Mycobacteria. By Pattisapu Rama Jogi Gangadharam. Published by CRC Press, Inc., 2000 Corporate Blvd, NW Boca Raton, FL 33431, 1984. Approx. 192 pp. Price: $56.00 prepub., U.S./$65.00 prepub., outside U.S. ISBN 0-8493-6574-0.

This book examines the origin and mechanisms of drug resistance, biological variations consequent to drug resistance, techniques for drug susceptibility testing, clinical significance of drug resistance, and the overall development of drug resistance.

The Pesticide Manual, Seventh Edition. Edited by C. R. Worthing. Published by The British Crop Protection Council, 'Shirley,' Westfields, Cradley, Malvern, Worcestershire WR13 5LP, England, 1983. Also available from the Commonwealth Agricultural Bureaux, Farnham House, Farnham Royal, Slough, SL2 3BN UK. Approx. 700 pp. ISBN 0-901436-77-1.

As in previous editions, the manual contains information on chemicals and microbial agents used as active components of products for control of weeds, crop pests, and diseases, as well as pests in public health and animal ecoparasites. The new edition contains about 590 chemicals, each described according to nomenclature and development, properties, uses, toxicology, formulations, and analysis.

Dangerous Properties of Industrial Materials, Sixth Edition. By N. I. Sax. Published by Van Nostrand Reinhold, 135 W 50th St, New York, NY 10020, 1984. 3152 pp. Price: $198.00.

This expanded and updated edition provides a comprehensive, single source reference to more than 18 000 industrial and laboratory materials, and also includes single-figure toxic hazard ratings for all the chemicals discussed. Also included are over 40 000 indexed synonyms, NIOSH and CAS identification

numbers, ready-to-use countermeasures, flammability ratings, explosion potentials, and new clinical data and references on human beings and experimental animals.

Van Nostrand Reinhold Encyclopedia of Chemistry, Fourth Edition. Edited by D. M. Considine and G. D. Considine. Published by Van Nostrand Reinhold, 135 W 50th St, New York, NY 10020, 1984. 1088 pp. Price: $89.50.

Fully revised and enlarged, this edition incorporates the advancements in chemistry achieved during the past decade, and updates the traditional aspects of the field. It features about 1300 alphabetically arranged editorial entries, each prepared by an expert in a given discipline. Also included are the industrial uses of natural and synthetic chemical solids, liquids, and gases; recent developments in chemical theory and practice; and numerous equations, structural formulas, and illustrations.

McGraw-Hill Dictionary of Science and Engineering. Edited by S. P. Parker. Published by McGraw-Hill Book Co., 1221 Avenue of the Americas, New York, NY 10020, 1984. 942 pp. Price: $32.50. ISBN 0-07-045483-3.

With more than 35 000 terms defined, this book focuses on the essential vocabulary of 100 different scientific and engineering disciplines, ranging from acoustics to zoology. Also provided are synonyms, acronyms, and abbreviations, listed separately as cross references.

Biotechnology of Industrial Antibiotics. Edited by E. J. Vandamme. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1984. 832 pp. Price: $105.00 (20% higher outside U.S. and Canada). ISBN 0-8247-7056-0.

Volume 22 of the Drugs and the Pharmaceutical Sciences Series examines the production of antibiotic compounds by fermentation biotechnology, and provides information on their microbiology, biochemistry, genetics, and engineering. Also included are compound properties, history, screening, strain improvement, biosynthesis, production process, product recovery, application, and mode of action.

Pharmaceutical Process Validation. Edited by B. T. Loftus and R. A. Nash. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1984. 304 pp. Price: $55.00 (20% higher outside U.S. and Canada). ISBN 0-8247-7164-8.

Designed to ensure compliance with FDA guidelines and objectives, Volume 23 of the Drugs and the Pharmaceutical Sciences Series provides a comprehensive background to the field; the hands-on experience of 19 expert contributors for procedures, guidelines, approaches, and terminology; and supplies information on both sterile and nonsterile processes, prospective and retrospective validation, analytical methods validation, statistical techniques, and solid dosage form validation.

Methods Involving Metal Ions and Complexes in Clinical Chemistry. Edited by H. Sigel. Published by Marcel Dekker, Inc., 270 Madison Ave, New York, NY 10016, 1984. 440 pp. Price: $75.00 (20% higher outside U.S. and Canada). ISBN 0-8247-7038-2.

Volume 16 of the Metal Ions in Biological Systems Series covers such topics as nutritional and immunological aspects of trace elements; therapeutic chelating agents; the determination of metals by various methods, the identification and quantification of phosphates, cannabinoids, and sulfanilamides via reactions with metal ions; and the use of gallium, indium, and technetium in nuclear medicine.

New Approaches in Liquid Chromatography. Edited by H. Kalasz. Published by Elsevier Science Publishers, PO Box 211, 1000AE Amsterdam, The Netherlands, 1984. Also available from Elsevier Science Publishing Co., Inc., PO Box 1663 Grand Central Station, New York, NY 10163. Approx. 300 pp. Price: $67.25 U.S. & Canada/Dfl. 175.00. ISBN 0-444-99642-7.

Volume 16 of the Analytical Chemistry Symposia Series contains full texts of the 23 papers presented at the 2nd Annual American–Eastern European Symposium, held June 16–18, 1982, in Szeged, Hungary. Topics covered include: HPLC of peptides and proteins; displacement chromatography using mathematical modeling of separation techniques; displacement mode of development on thin-layer plates and the effect of length of development; sample size and position of spots in the separation of structurally related compounds; characterization of the stationary phases for HPLC; optimization of HPLC and gel chromatography; and results of analytical and preparative separation of amino acids, peptides, and proteins.

HIGHLIGHTS

The 98th Annual International Meeting

OF THE AOAC

Shoreham Hotel, Washington, D.C. October 28–November 2, 1984

1884 Centennial Meeting 1984

October 25–27 • Joint IUPAC-AOAC International Symposium on Harmonization of Collaborative Studies

October 27–28 • Quality Assurance for Analytical Laboratories—Short Course

October 28 • Botanic Gardens Reception

October 29 • Opening Session with Celebrated Speakers: Nobel Laureate Linus Pauling and former FDA General Counsel Peter Barton Hutt • Education in Modern Analytical Chemistry—Symposium • Sampling and Analysis of Fertilizers—Symposium (also Tues.) • New Equipment and Memorabilia Exhibits (also Tues. and Wed.) • Industry Night—a New Products Showcase and Mixer

October 30 • Microcomputer Workshop • Analysis of Pesticide Products for Impurities and Degradatior Products—Symposium • Quality Assurance/Quality Control for Analytical Laboratories—Symposium (alsc Wed.) • Reception and Gala Centennial Banquet

October 31 • Physical-Chemical Methods—Antibiotic Residues in Tissues and Milk of Food Producing Animals—Symposium • Environmental Toxicology anc Chemical Analysis—Symposium (also Thurs. and Fri.)

November 1 • Storage, Handling & Disposal of Hazardous Chemicals: Laboratory Safety—Symposium • Chromatographic Methods in Drug Analysis of Body Fluids—Symposium

November 2 • Disinfectants Workshop

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AOAC CENTENNIAL MEETING — OCTOBER 28–NOVEMBER 2, 1984

(Advance Registration Deadline: October 10, 1984)

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Arlington, VA USA 22209

For more information, contact Margaret Ridgell at above address or at (703) 522-3032 in U.S., +1-703-522-3032 outside U.S.

(Photocopy this form for additional advance registrations.

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At The Centennial Meeting—
The 98th Annual AOAC International Meeting

October 29-November 2, 1984
Shoreham Hotel, Washington, DC

Eight Informative Symposia

Analysis of Pesticide Products for Impurities and Degradation Materials	Chairmen: James Launer, Oregon Department of Agriculture Alan R. Hanks, Purdue University October 30, 1984
Sampling and Analysis of Fertilizers	Chairmen: Robert C. Rund, Purdue University Frank J. Johnson, Tennessee Valley Authority October 29-30, 1984
Quality Assurance/Quality Control for Analytical Laboratories	I—*Administration* II—*Laboratory Quality Control* III—*Collaborative Studies & Statistics* Chairman: Keith A. McCully, Health & Welfare Canada October 30-31, 1984
Education in Modern Analytical Chemistry	Chairman: Henry Freiser, University of Arizona October 29, 1984
Physical-Chemical Methods for Determining Antibiotic Residues in Tissues and Milk of Food Producing Animals	Chairmen: William A. Moats, US Department of Agriculture Badar Shaikh, US Food and Drug Administration October 31, 1984
Environmental Toxicology and Chemical Analysis	Chairmen: David Stallings, US Department of Interior Warren Crummett, Dow Chemical Co. October 31-November 2, 1984
Chromatographic Methods in Drug Analysis of Body Fluids	Chairman: Pokar Kabra, University of California, San Francisco November 1, 1984
Storage, Handling, & Disposal of Hazardous Chemicals: A Laboratory Safety Symposium	Chairman: Robert Bianchi, Drug Enforcement Administration November 1, 1984

For more information or to register, contact Meeting Manager,
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A New Handbook on How to Set Up or Improve Laboratory Quality Assurance Programs

Quality Assurance Principles for Analytical Laboratories

by Frederick M. Garfield
former Assistant Administrator
U.S. Drug Enforcement Administration

This handbook provides essential information needed to design, document, implement, or improve a laboratory quality assurance program . . . A program that can enable you to document the credibility of your laboratory's analytical data.

The handbook also provides a rational and solidly based justification for commitment of resources to improved laboratory operation.

Drawing from published principles, practices, guidelines, and procedures, the book brings together the experiences of experts who have developed and implemented *successful* "QA" programs.

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VII. Audit Procedures
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IX. Laboratory Accreditation Programs and Good Laboratory Practices Regulations

Appendices—

A. Quality Assurance Publications and Programs
B. Forms Used by U.S. Federal Agencies
C. Instrument Performance Checks
D. Control Charts
E. FDA Audit Measure Procedures
F. Safety Publications
G. Glossary

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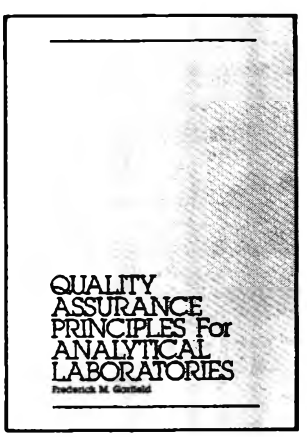

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FOR YOUR INFORMATION

AOAC to Hold Centennial International Meeting

The Association of Official Analytical Chemists (AOAC), founded in 1884, will highlight its centennial celebration with the 98th Annual International Meeting and Exposition, to be held October 28–November 2, 1984, at the Shoreham Hotel, Washington, DC.

Throughout the years, AOAC has continued to monitor the evolution of methods of analysis, which have expanded to include many new materials; to promote uniformity and reliability in the statement of analytical results; to promote, conduct, and encourage research in agriculture, public health, and regulatory control of commodities in these fields; and to afford opportunity for discussion of matters of interest to scientists engaged in relevant pursuits.

The Centennial Meeting will feature noted international speakers, including: Dr. Linus Pauling, Nobel Laureate; Peter Barton Hutt, former General Counsel of the Food and Drug Administration; and Dr. Cyril Ponnamperuma, Director of the University of Maryland Laboratory of Chemical Evolution.

The technical program will include 8 symposia: Analysis of Pesticide Products for Impurities and Degradation Materials, October 29–30; Sampling and Analysis of Fertilizers, October 29–30; Quality Assurance/Quality Control for Analytical Laboratories, October 30–31; Education for Modern Analytical Chemistry, October 29; Physical-Chemical Methods for Determining Antibiotic Residues in Tissues and Milk of Food Producing Animals, October 31; Environmental Toxicology and Chemical Analysis, October 31–November 2; Storage, Handling, and Disposal of Hazardous Chemicals: A Laboratory Safety Symposium, November 1; and Chromatographic Methods in Drug Analysis of Body Fluids, November 1.

The balance of the technical program will consist of more than 200 presentations of papers on new techniques, methods, and instrumentation for the analysis of foods, drugs, pesticides, cosmetics, feeds, fertilizers, mycotoxins, beverages, colors, forensic science materials, hazardous substances, vitamins, water and air pollutants, microbiological and extraneous material contamination of foods, and related subjects.

A number of international organizations, invited to join the AOAC celebration, will meet during the week of October 22–27: a joint International Union of Pure and Applied Chemistry (IUPAC)–AOAC International Symposium on Harmonization of Collaborative Studies, a Collaborative International Pesticide Analytical Council (CIPAC) meeting, International Dairy Federation (IDF) Chemical Week, and meetings of International Organization for Standardization Technical Committees 134 (ISO/TC 134-Fertilizers) and 34 (ISO/TC 34/SC7-Spices).

More than 75 suppliers will exhibit the latest in analytical equipment and services. At Exhibitors Night, October 29, exhibitors will make short presentations on new products.

The 2-day AOAC short course, Quality Assurance for Analytical Laboratories—Plan Development and Management, is scheduled for October 27 and 28, preceding the Annual Meeting to enable course registrants to attend the meeting. Short course registration is limited to 30 persons and is separate from meeting registration. Course fees are $300.00 for members and $335.00 for nonmembers.

Two workshops are presently scheduled—an all-day, walk-in workshop on using the microcomputer to integrate information into scientific reports and publications (October 30), and a workshop in the evening on analytical techniques.

The opening reception will be held at the U.S. Botanical Gardens on Sunday evening, October 28. The Centennial Banquet will be on Tuesday evening, October 30.

Advance registration fees for the meeting are $35.00 (Members)/$45.00 (Nonmembers) for one day and $60.00 (Members)/$85.00 (Nonmembers) for the full meeting. On-site registration for one day is $50.00 (Members)/$60.00 (Nonmembers) and $75.00 (Members)/$100.00 (Nonmembers) for the full meeting.

For hotel reservations, contact the Shoreham Hotel, Calvert St and Connecticut Ave, NW, Washington, DC 20008; 202/234-0700.

For further details, contact Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209; 703/522-3032.

Interim Methods Adopted

The following methods have been adopted interim first action by approval of the appropriate General Referee, Committee on Official Methods, and Chairman of the Official Methods Board: Atomic Absorption Spectrometric Determination of Tin in Canned Foods, using Nitric Acid-Hydrochloric Acid Digestion and Nitrous Oxide-Acetylene Flame, by Robert W. Dabeka, Arthur E. McKenzie, Health and Welfare Canada, and Richard Albert, Food and Drug Administration; Luke et al. Method for Multipesticide Residues in Fruits and Vegetables, by Leon D. Sawyer, Food and Drug Administration; Atomic Absorption Spectrometric Determination of Copper in Liver, by David L. Osheim and P. Frank Ross, Ames National Veterinary Services Labs; Color Classification of Honey by Lovibond 2000 Comparator, by Jonathan W. White, Honeytech, Inc.; Liquid Chromatographic Determination of Methyldopa and Methyldopa-Thiazine Combinations in Tablets, by Susan Ting, Food and Drug Administration; and Liquid Chromatographic Determination of Allopurinol in Tablets, by Donald Shostak, Food and Drug Administration.

These methods will be recommended for vote and adoption by the membership at the AOAC Centennial International Meeting, October 28–November 2, 1984.

Check Sample Service

The American Association of Cereal Chemists (AACC) announces a new Check Sample Program for ethylene dibromide (EDB). The special sample will be issued monthly and will contain 2 products: a whole grain product, such as wheat, corn, oats, or rice, and a flour or mix.

To ensure uniformity on delivery, samples will be packed in ½ pint Ball jars sealed with a lid and rings, packed on dry ice, and shipped via UPS Blue Label, with an enclosed reporting form.

Subscribers are cautioned to keep samples frozen until analysis and to submit their results in parts per billion to AACC no later than 10 days after receiving the sample to achieve accurate results. Results may be mailed or phoned in to AACC. Although optional, subscribers will be asked to indicate the method of EDB analysis used. Subscribers are identified only by collaborator number.
for galley 242

The price for the 12 monthly samples is $600.00. The first sample was shipped July 1, 1984. For more information, contact: AACC Check Sample, 3340 Pilot Knob Rd, St. Paul, MN 55121; 612/454-7250.

Short Courses

The American Association of Cereal Chemists (AACC) announces 5 short courses: (1) Food Fortification: Practical Aspects of Fortifying Foods with Vitamins, Minerals and Proteins, October 4–5, 1984, Holiday Inn, Minneapolis, MN. This course will provide food technologists, product managers, and management personnel who are involved in the formulation, development, and marketing of foods with an understanding of why and how vitamins, minerals, and protein can be added to foods.

(2) Gum Chemistry and Technology in the Food Industry, November 7–9, 1984, Holiday Inn, Chicago City Center, Chicago, IL. This course will provide food chemists, scientists, and production and related managerial personnel who are involved in the formulation, development, and manufacture of new and improved foods with an understanding of the role of polysaccharide gums in processed food products.

(3) Starch: Structure, Properties, and Food Uses, December 6–7, 1984, Holiday Inn O'Hare Kennedy, Chicago, IL. This course will provide food and industry personnel with an understanding of the chemical and physical properties of starches and their uses in food.

(4) Milling for Cereal Chemists, January 1985, Kansas State University, Manhattan, KS. (5) Introduction to Cereal Chemistry and Technology, May 1985, Minneapolis, MN. For more information, contact: Dotty Ginsburg, AACC, 3340 Pilot Knob Rd, St. Paul, MN 55121; 612/454-7250.

Editors Seek News about Rare Tests

The American Association for Clinical Chemistry (AACC) plans to publish the second edition of the *Directory of Rare Analyses* in late 1984 or early 1985. The directory is a compilation of clinical laboratories that perform unusual tests, including those tests not already performed by two or more of the country's major reference laboratories.

Readers who know of individuals or laboratories performing unusual tests are asked to inform the Directory's editors, Dr Jocelyn Hicks and Dr Donald Young, by contacting Marilyn Werkheiser, AACC, 1725 K St, NW, Suite 1010, Washington, DC 20006; 202/857-0717.

Meetings

September 30–October 4, 1984: American Association of Cereal Chemists 69th Annual Meeting, Hyatt Regency, Minneapolis, MN. The technical program will include the following symposia: Complex Carbohydrates—Their Role in a Healthy Diet; Protein Improvement in Cereals and Oilseeds Through Traditional and Modern Genetic Approaches; Starch Properties in Processed Foods; Bread Baking—Research Findings and Practical Consequences; Water Activity—Influence on Cereal Foods; Wheat Industry Council Symposium; and the Good Housekeeping Institute Consumer Concerns Symposium. Contact: AACC Annual Meeting, 3340 Pilot Knob Rd, St. Paul, MN 55121; 612/454-7250.

October 11–13, 1984: Symposium on the Diagnosis of Mycotoxicosis of Importance in the United States and Japan, National Animal Disease Center, PO Box 70, Dayton Ave, Ames, IA, sponsored by the United States–Japan Cooperative Program, the Development and Utilization of Natural Resources Joint Panel on Toxic Microorganisms, and the National Animal Disease Center. Contact: John L. Richard, PO Box 70, Dayton Ave, Ames, IA 50010.

October 26–27, 1984: Northeastern Association of Forensic Scientists 10th Annual Meeting, Long Island Marriott Hotel, Uniondale, New York. Sessions will include: drugs, criminalistics, toxicology, serology, forensic paint analysis, computer assisted instrumentation, laboratory safety, and clandestine laboratory synthesis. Contact: Jeffrey M. Weber, Drug Enforcement Administration, Northeast Laboratory, 555 West 57th St, Suite 1886, New York, NY 10019; 212/399-5137.

October 28–November 2, 1984: AOAC Centennial International Meeting, Shoreham Hotel, Washington, DC. Contact: Margaret Ridgell, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209; 703/522-3032.

October 29–30, 1984: AOAC Symposium on Sampling and Analysis of Fertilizers, Shoreham Hotel, Washington, DC. One of the events planned for the centennial celebration, this symposium will feature international speakers, addressing a wide variety of topics including development and influence of AOAC fertilizer methods throughout the world on the measurement and effects of physical properties on sampling and analyses; analytical automation; laboratory computerization; and statistical quality control. Contact: Robert C. Rund, Purdue University, Dept of Biochemistry, West Lafayette, IN 47907; 317/494-1492. Frank J. Johnson, TVA, Natl Fertilizer Dev. Center, Muscle Shoals, AL 35660; 205/386-2543.

April 9–11, 1985: AOAC 10th Annual Spring Training Workshop, Sheraton Hotel, Dallas, TX. The program will contain sessions on a variety of topics, including the environment, foods, feeds, drugs, and instrumentation. Contact: M. Virginia Gibson, FDA, 332 Bryan, Dallas, TX 75204; 214/767-0312 and Molly Ready, Alcon Labs, 6201 S Freeway, Fort Worth, TX 76134; 817/293-0450.

April 28–May 3, 1985: American Chemical Society Division of Analytical Chemistry Symposium on Analytical Methods in Forensic Chemistry and Toxicology in conjunction with ACS 189th National Meeting, Miami, FL. The scientific program of the symposium will comprise invited lectures and contributed papers dealing with the following topics: novel optical and spectroscopic methods, analytical separation and chromatographic methods, electroanalytical methods, biological and immunological methods, automated systems, computer aided methods, luminescences, electrophoresis, drug analysis, explosive analysis, gunshot residue, arson and bloodstain investigations, trace evidence detection, and other recent advances in forensic analytical chemistry and analytical toxicology.

ACS invites individuals and groups to submit for consideration abstracts of papers that are reviews or original research. Abstracts of 150 words on ACS standard abstract forms should be sent to M. H. Ho, Department of Chemistry, University of Alabama in Birmingham, Birmingham, AL 35294; 205/934-4747, by November 30, 1985. The proceedings will be published as a hard-cover book in the ACS Symposium Series.

April 29–May 3, 1985: American Chemical Society International Symposium on Biological Monitoring of Exposure to Metals and Inorganic Compounds, Miami, FL. This symposium will assess the directions of biological monitoring and emphasize metals, inorganic and organometallic compounds, and their metabolites. Topics to be covered include: analytical methods; metabolism and metabolic indicators; conditions, limitations, and reliability of biological monitoring; relationship to ambient monitoring and medical screening; correlation and interpretation of results; and genetic markers of toxicity. Authors wishing to contribute

papers must submit 150-word abstracts to H. K. Dillon, Southern Research Institute, 2000 9th Ave, S, PO Box 55305, Birmingham, AL 35255-5305; 205/323-6592 by November 30, 1984. Publication of the proceedings is planned.

New Private Sustaining Members

AOAC welcomes 2 new Private Sustaining Members to the growing list of firms aware of the need to support an independent methods validation association: Heublein Wines, 12667 Rd. 24, Madera, CA; and Royal Iceland Corp., PO Box 5715, Berkeley, CA.

YES, the 10th Annual AOAC Spring Training Workshop will be held in DALLAS

APRIL 8-11, 1985

at the New Sheraton-Plaza of the Americas Complex where there are 20 restaurants, shops, an ice skating rink, and an art museum.

For more information contact:
M. Virginia Gipson, Food and Drug Administration, 3032 Bryan, Dallas, TX 75204, (214) 767-0309 or
Molly Ready, Alcon Laboratories, 6201 S. Freeway, Fort Worth, TX 76134, (817) 293-0450

INSTRUCTIONS TO AUTHORS

Scope of Articles

The Journal of the AOAC will publish articles that present, within the fields of interest of the Association *(a)* unpublished original research; *(b)* new methods; *(c)* further studies of previously published methods; *(d)* background work leading to development of methods; *(e)* compilations of authentic data; *(f)* technical communications, cautionary notes, and comments on techniques, apparatus, and reagents; *(g)* invited reviews of methodology in special fields. The scope broadly encompasses the development and validation of analytical procedures pertaining to both the physical and biological sciences related to agriculture, public health and safety, consumer protection, and quality of the environment. Emphasis is focused on research and development to test and adopt precise, accurate, and sensitive methods for the analysis of foods, food additives, supplements and contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment. Compilations of authentic data include monitoring data of pesticide, metal, and industrial chemical residues in food, tissues, and the environment. All articles are reviewed for scientific content and appropriateness.

Preparation of Manuscript

Authors must submit 3 copies of the complete manuscript, including tables and illustrations, to AOAC, 1111 N 19th St, Arlington, VA. The manuscript is to be typewritten on one side of white bond paper, 8½ × 11 inches, with page margins of 1 inch, and **double-spaced** throughout (i.e. title, authors' names and addresses, footnotes, tables, references, captions for illustrations, and the text itself). Tables are to be typed on separate sheets, *not* interspersed through the manuscript. Drawings and photographs should be mounted apart from the text or submitted as separate items.

Style and Format

The text should be written in clear, concise, grammatical English. Unusual abbreviations should be employed as little as possible and must always be defined the first time they appear. Titles of articles should be specific and descriptive. Full first names, middle initial (if any), and last names of authors should be given. The address of the institution (including zip code) from which the paper is submitted should be given and should be in a form to which inquiries, proofs, and requests for reprints can be sent. Information supplementing the title and names and addresses should be given as footnotes.

Methods, Results and/or Discussion, Acknowledgments, and Recommendations (applicable to reports of General and Associate Referees) should be placed in sections under appropriate headings.

Abstracts: Each manuscript should be accompanied by a concise abstract (not more than 200 words). The abstract should provide specific information rather than generalized statements.

Introduction: Each article should include a statement on why the work was done, the previous work done, and the use of the compound being studied.

Methods: Methods should be written in imperative style, i.e., "Add 10 mL . . . Heat to boiling . . . Read in spectrophotometer." Special reagents and apparatus should be separated from the details of the procedure and placed in sections with appropriate headings; however, common reagents and apparatus or those which require no special preparation or assembly, need not be listed separately. Reagents and apparatus listed should be described in generic terms and in terms of performance; use of brand names should be avoided. Hazardous and/or carcinogenic chemicals should be noted. Any very long, detailed operation can be given in a separate section with an appropriate heading (e.g., Preparation of Sample; Extraction and Cleanup; Preparation of Standard Curve). Any necessary calculations should be included; number of significant figures must reflect the accuracy of the method. Wherever possible, metric units should be used for measurements or quantities.

Tables: All tables must be cited in the text consecutively. Tables are numbered by arabic numbers, and every table must have a descriptive title, sufficient so that the table can stand by itself without reference to the text. Every vertical column in the table should have a heading; abbreviations may be used freely in the headings to save space, but should be self-evident or must be explained in footnotes. Footnotes to both the headings and the body of the table are indicated by lower case letters in alphabetical order; these letters should be underscored and raised above the line of type. Horizontal rules should be used sparingly; however, they are used to bound the table at top and bottom and to divide the heads from the columns. Authors should refer to recent issues of the Journal for acceptable format of tables.

Illustrations: Illustrations, or figures, may be submitted as original drawings or photographs; photocopies are acceptable for the two review copies but not for the printer's copy. All figures must be cited in the text consecutively. Figures are numbered by arabic numbers, and all figures must be accompanied by descriptive captions, typed on one (or more) separate sheets, *not* on the figure itself. The figure should be identified by number on the back by a soft pencil or (preferably) a gummed label.

Drawings should be submitted either as the original drawing or a good glossy photograph; photocopies, multiliths, Verifax copies, Xerox copies, etc. are *not* acceptable. Drawings should be done in black India ink (ordinary blue or blue-black ink is not acceptable) or with drafting tape on white tracing paper or tracing cloth or on "fade-out" graph paper (ordinary graph paper ruled with green or dark blue ink is not acceptable). Lettering should be done with a Leroy lettering set, press-on lettering, or a similar device; freehand or typewritten lettering is not acceptable. Values for ordinate and abscissa should be given, with proper identification conforming to Journal style (example: wavelength, nm), at the sides and bottom of the figure. Lettering or numbering on the face of the figure itself should be kept at a minimum; supplementary information should be given in the caption. Several curves on the same figure should be identified by simple symbols, such as letters or numbers, and the proper identification or explanation given in the caption. Letters and numbers should be large enough to allow reduction to journal page or column size. The Journal does not publish straight line calibration curves; this information can be stated in the text.

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References to journal articles must include the following information: last names and at least one initial of *all* authors (*not* just the senior author); year of publication, enclosed in parentheses; title of journal, abbreviated according to accepted *Chemical Abstracts* style; volume number; numbers of first and last pages. References to books, bulletins, pamphlets, etc. must include the following information: last names and initials of authors or editors; year of publication, enclosed in parentheses; full title of book; volume number or edition (unless it is the first edition); publisher; city of publication; numbers of pertinent pages, chapter, or section. Citation to private communications or unpublished data should be included in the text, *not* in the list of references.

Spectrophotometric, gas chromatographic, and liquid chromatographic nomenclature should follow the practice recommended by the American Society for Testing and Materials.

Rev. 1/84

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The Association The primary objective of the Association of Official Analytical Chemists (AOAC) is to obtain, improve, develop, test, and adopt uniform, precise, and accurate methods for the analysis of foods, vitamins, food additives, pesticides, drugs, cosmetics, plants, feeds, fertilizers, hazardous substances, air, water, and any other products, substances, or phenomena affecting the public health and safety, the economic protection of the consumer, or the protection of the quality of the environment; to promote uniformity and reliability in the statement of analytical results; to promote, conduct, and encourage research in the analytical sciences related to agriculture, public health, and regulatory control of commodities in these fields; and to afford opportunity for the discussion of matters of interest to scientists engaged in relevant pursuits.

Membership Membership in AOAC is open to all interested persons worldwide. Sustaining memberships are available to any government agency or private company interested in supporting an independent methods validation program.

The Journal The Journal of the Association of Official Analytical Chemists is published by AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209. The Journal is issued six times a year in January, March, May, July, September, and November. Each volume will contain approximately 1400 pages. The scope of the Journal broadly encompasses the development and validation of analytical procedures pertaining to both the physical and biological sciences related to agriculture, public health and safety, consumer protection, and the quality of the environment. Emphasis is focused on research and development to test and adopt precise, accurate, and sensitive methods for the analysis of foods, food additives and supplements, contaminants, cosmetics, drugs, toxins, hazardous substances, pesticides, feeds, fertilizers, and the environment.

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A CENTURY OF ANALYTICAL EXCELLENCE

The History of Feed Analysis, as Chronicled in the Development of AOAC Official Methods, 1884–1984

VALVA C. MIDKIFF
Retired, University of Kentucky, College of Agriculture, Lexington, KY 40546-0064

In the early part of the nineteenth century, chemists in Germany, France, and England were beginning to experiment in the field of agriculture. Agricultural experiment stations began to study soils, fertilizers, and related topics. German agricultural chemists recognized the necessity for uniformity of methods and for testing and improving them.

The largest business in the United States at the time (about 1850–1880) was agriculture. Because there were signs of soil deterioration through continuous cultivation, agricultural chemists also became interested in the constituents of soils, soil erosion, and fertilizers. The states began to appoint legislative committees on agriculture, to make geological surveys, to start agricultural publications, and to form agricultural societies; chemists began to preach the importance of scientific agriculture, particularly the use of fertilizers to increase crop production.

At this time, there were no uniform methods of sampling and analyzing various brands of fertilizers. State laboratories and manufacturers of fertilizer were using different methods, which led to widely varying results and regulatory confusion.

On May 20, 1880, J. T. Henderson, Commissioner of Agriculture for Georgia, mailed a circular letter to several commissioners of agriculture, representatives of state boards of agriculture, state chemists, and professors of chemistry in state universities and state agricultural colleges to suggest a convention to discuss fertilizer analysis. A meeting was held in Washington, DC, on July 28, 1880, "to consider the importance of securing such uniformity of methods in determining by chemical analysis the percentage of valuable ingredients in commercial fertilizers, as well as give more uniform, and hence more satisfactory, results" (1).

Additional meetings were held in Boston on August 27, 1880, and in Cincinnati on August 18, 1881. Because of continued unsatisfactory conditions, Judge J. T. Henderson, president of the Agriculture Chemists, called for a fourth meeting in Atlanta, on May 15, 1884. This convention appointed a committee to develop a permanent organization of agricultural chemists. On September 9, 1884, in Philadelphia, the Committee on Organization submitted its report in the form of a constitution, which was adopted, and this became the first meeting of the Association of Official Agricultural Chemists.

From the beginning, the purpose of AOAC has been to bring together methods, test them through collaborative study, and publish the approved official methods for general use. The uniqueness of the AOAC procedure is the collaborative study. This cooperative effort of many laboratories working under a variety of conditions really tests the ruggedness of a method. One could not possibly get the quantity and quality of data, such as inter- and intralaboratory differences, except through collaborative study. There is no substitute for it.

Of course, these reliable, validated methods are not sacred. As materials and conditions change, the analytical methods must also change. Official methods are continually evaluated to keep analyses in step with new products and new compositions which may need new methods or modifications of present methods. The AOAC mechanism is flexible enough to make these changes.

Because AOAC methods are thoroughly studied, collaboratively tested, statistically treated, and evaluated over a period of time, they have the prestige and authority recognized in the courts. These methods are used throughout the world for protection of the consumer and honest manufacturer of materials.

In the beginning, AOAC members tested only fertilizers and, therefore, chose the name Association of Official Agricultural Chemists. Now the scope of AOAC has become so broad that in 1965 a more appropriate name was chosen—Association of Official Analytical Chemists.

For the first few years after 1884, the Association studied methods for the determination of nitrogen, phosphoric acid, and potash in fertilizers, exclusively. In 1886, the Association started testing feedstuffs, such as corn, cottonseed meal, linseed oil meal, hay, and, particularly, fodders for protein, ether extract or fat, fiber, moisture, ash, and nitrogen-free extract. Fodder analysis was very popular at this time. Apparently, little cooperative testing and unification of methods had been done in Germany, and none had been done on fodders. In his address in 1893, the AOAC president said, "except for the examination of commercial fertilizers, the methods generally used for fodder analysis use more time of the agricultural chemists than any other line." Feedstuffs can be very complex mixtures. In 1896, chemists had difficulties even with single products, as stated in the address of the president: "The matter of the analysis of foods and feedstuffs, as shown by the experience of this association, is one of the most difficult questions connected with the work of this organization." As we move through the years, feedstuffs become increasingly more complex and more interesting for the chemist to analyze.

Protein

Protein is one of the most important constituents of feeds. In determining protein, we actually measure the nitrogen content and multiply it by the factor 6.25. Because feeds do not contain nitrates and are mostly organic materials, the methodologies for protein in feeds are different from those in fertilizers.

Some of the older procedures for nitrogen were the Ruffle, soda-lime, cupric oxide or absolute, and Kjeldahl. Except for the Kjeldahl procedure, these were very tedious and time consuming. One of the first official methods for determination

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of nitrogen was the Dumas method, where gaseous nitrogen is evolved, collected, measured, and reduced to standard conditions for calculation of percentage of protein.

The Kjeldahl method was first introduced in 1883 (2). Three years later, agricultural chemists had become interested in this method because it was easier and more rapid than the Dumas or Ruffle method. Briefly, the sample was digested in 20 mL sulfuric acid with 0.7 g mercuric oxide until the solution was clear, usually 1½ h. Potassium permanganate was added dropwise until the liquid remained purple; then the solution was cooled and transferred to a distillation flask. Zinc granules, potassium sulfide, and alkali were added, the mixture was distilled into standard hydrochloric acid, and back-titrated with standard ammonia. Heat was supplied by Bunsen burners for both digestion and distillation. Wilfarth was the first to advocate the use of mercury in Kjeldahl digestions (3). Mercuric oxide was not used in the original work of Kjeldahl. In 1887, this was the official method for determining nitrogen in cattle feed. In the 1890 annual meeting report on nitrogen, Van Slyke said that he had used the J. W. Gunning method (4), in which the author used one part potassium sulfate and two parts strong sulfuric acid on wheat middlings, flour, and other organic substances. Results compared closely with the official method. No potassium permanganate, no form of mercury, and no potassium sulfide were used.

A collaborative study of the Kjeldahl and the Gunning methods on bone and cottonseed meal samples gave practically the same results for both methods. The Gunning method became an official alternative for analysis of organic materials (cattle feed). In the 1893 annual report on nitrogen, the recommendations were as follows:

1. That the Gunning method be put on full equality with the Kjeldahl method.
2. That the strength of standard acid be 0.2N and the alkali be 0.1N.
3. That sulfuric acid be standardized by $BaCl_2$ and that sodium hydroxide as well as standard hydrochloric acid and ammonia be official.

Some chemists preferred standard sodium hydroxide to standard ammonium hydroxide (ammonia) because it contained no volatile constituents.

Through the years, various methods such as the Dumas, soda-lime, and Kjeldahl had been used. By 1920, the Kjeldahl method seemed to be used almost universally by feed control chemists. However, protein results on some materials by some analysts were often not in good agreement. It was thought that analysts were not careful in carrying out minute details of the method. Little had been published on the results of investigations where details of the method had been studied for causes of discrepancies. To study various parts of the apparatus and the digestion step, an investigation (5) was made using cottonseed meal, because it is particularly difficult to analyze, and pure ammonia salts. Ammonium chloride was used in a study of the following aspects:

1. Efficiency of connecting bulbs and of distillation apparatus used.
2. Effect of varying quantities of standard acid and water in the receiving flasks and effect of varying the quantities of distillate.
3. Quantity of distillate necessary when large amounts of nitrogen are present.
4. Effect of loss of sulfuric acid and of digestion with sulfuric acid alone.
5. Effect of using a short-neck flask and of using a guard with a long-neck flask.
6. Effect of overheating, allowing a strong flame to strike the flask above the acid.
7. Influence of time and of use of potassium sulfide in connection with various reagents used in the protein determination.
8. Effect of mercuric oxide, potassium permanganate, and copper sulfate reagents, and of the quantity of sample used in the protein determination.

A summary of the findings was as follows:

1. The use of an efficient distillation bulb is important.
2. The use of a guard in digestion is essential.
3. The use of potassium permanganate is at least unnecessary.
4. The reagents, in addition to sulfuric acid, which are most time efficient are combined potassium or sodium sulfate, mercuric oxide, and potassium sulfide.
5. Copper sulfate may be used in place of mercuric oxide and potassium sulfide if digestion time is lengthened.
6. The digestion time after clearing is important and should be determined for each kind of substance that is examined. In most instances, 3 h digestion is sufficient.

Having made this investigation, the authors suggested the following method which, if carefully followed in every respect, would give satisfactory results for cottonseed meal or similar materials:

Grind the sample to such a fineness that it will pass through a 30 mesh sieve, or a sieve having round 1 mm openings. Place 2 g of this thoroughly mixed sample in 500 mL digestion flask, add 0.5–0.7 g mercuric oxide or its equivalent of metallic mercury, 5–10 g potassium or sodium sulfate, and shake the flask until the contents are well mixed. Add 30 mL sulfuric acid and again shake until the acid and dry material in the flask are perfectly homogeneous; this mixture should be practically free from lumps. Place the flask in an inclined position, using a guard having a hole for the flask of such size that the flask can never be exposed to the bare flame above the acid at any time during digestion, and heat with a very low flame until the frothing ceases. Gradually increase the flame until the liquid boils briskly, using a flame with air so regulated that it gives a sharp, pointed, blue flame. Continue this heating until the solution is entirely clear; this should require 1–1½ h. Decrease the heat until the liquid boils gently and continue this digestion for 3 h. Let cool and add an excess of saturated sodium hydroxide solution (50–60 mL), permitting it to run down the side of the flask so that it does not mix with the acid solution. Add a few pieces of granulated zinc and connect the flask with the distillation apparatus. This apparatus must be equipped with the most efficient form of Kjeldahl connecting bulb, inserted between distillation flask and condenser. Mix the contents by shaking and distill about 150 mL into very accurately measured standard acid, using an excess of acid necessary to hold all the ammonia.

If it is desired to use copper sulfate in place of mercuric oxide, substitute 0.3–0.5 g copper sulfate for the mercury, digest for 6 h after the solution becomes clear, and omit the potassium sulfide.

The above method was used with slight modification for about 30 years. From what we know now, the digestion step was the critical part because of the salt:acid ratio and the lack of sufficient, even heat in the digestion.

In a contributed paper, "Kjeldahl Determination of Nitrogen in Refractory Materials" (6), nicotinic acid was used to evaluate catalysts, particularly selenium with mercuric oxide, and to determine the amount of potassium sulfate for the digest. In the discussion of the experiments, the time of digestion and the amounts of potassium sulfate and mercuric

oxide had an interrelated effect on the loss of nitrogen in presence of selenium. Selenium as an additional catalyst was not recommended. Because potassium sulfate is usually measured by volume, it is possible, when trying to measure 15 g, to actually measure 11 g or 13 g. This can affect the results remarkably.

The following year, a study (7) showed that the great discrepancies of results may be attributed principally to the boiling temperatures or insufficient heat, particularly with refractory materials.

A collaborative study of the Dumas method using the Coleman nitrogen analyzer was conducted, recommended, and adopted official first action (8). The sample weights used ranged from 50 to 300 mg, depending on the kinds of materials. The weak point in this procedure is the impracticality of grinding the sample fine enough and thoroughly mixing it to obtain, in the small weight called for, a representative aliquot for most samples routinely analyzed in a control laboratory.

Since 1970, much has been written about semiautomated and automated nitrogen methods for feeds, using the block digestor, Technicon Kjeldahl analyzer, Kjel-Foss analyzer, stopped-flow analyzer, and combinations of these. Ammonia is determined by the indophenol blue or ammonia salicylate reaction. All these techniques compare well with the official manual Kjeldahl procedure. They save time and material costs but the instruments are usually expensive, difficult to set up and calibrate, and not as versatile as the standard manual Kjeldahl method.

By 1941, research had shown that ruminants could use ammoniacal and urea nitrogen as partial substitutes for protein. It was apparent that a method of analysis would be needed if these ingredients were to be used in cattle feeds. A method was developed, collaboratively studied, modified, and adopted official (9). Briefly, the method is as follows:

Place the samples in a Kjeldahl digestion flask, add water and urease solution, stopper, and let stand 1 h at room temperature. Rinse the stopper, add MgO, a small amount of $CaCl_2$, and defoamant, distill into a measured quantity of standard acid, and titrate with standard alkali.

A colorimetric procedure for urea in feeds and the AOAC urease method were studied collaboratively in 1961 (10). The reaction of urea and *p*-dimethylaminobenzaldehyde (DMAB) was the basis of the colorimetric procedure. At this time the DMAB method did not have the accuracy and precision of the official method. Later (1967), the method was improved with regard to sampling, color reading (420 nm instead of 440 nm), and calibration (reference standard analyzed with each set of samples). A collaborative study compared the official enzymatic method with the colorimetric method; results were satisfactory, and the colorimetric method was adopted (11). It is still in use today.

Fat or Ether Extract

Ether extract (fat) is one of the earliest determinations. In 1887, the first samples sent to chemists in experiment stations were hay, bran, and cottonseed meal with instructions to use the methods of preference. Some chemists used Squibbs ether, others used ordinary washed ether; some used Soxhlet self-siphoning extractors, some used continuous extractors, some used a few hours extraction, and others used up to 3 or 4 days extraction. The results varied as much as 2% from an average total of 3–4%.

By 1891, the method was as follows: Dry 2 or 3 g of the substance 4 h by heating fully to the temperature of boiling water in a current of dry hydrogen; extract 16 h with specially prepared anhydrous and alcohol-free ether. Dry the extract to constant weight by exposure to the full heat of boiling water. If the feed contains molasses, wash with water, filter, dry, and then extract with ether.

In the early 1930s, dried milk products were used in feeds. To determine fat in these materials, the Roese-Gottlieb tentative, the Roese-Gottlieb modified, and the acid hydrolysis methods were studied. The Roese-Gottlieb modified method was adopted because it was simple and convenient and gave good comparative results (12).

In the late 1930s, fish meal was used extensively in feeds. Fat content, depending on the type of raw materials and the method of processing used in their manufacture, seemed to vary widely. Testing laboratories added to the confusion by using different fat solvents. Industry asked AOAC to study the problem.

Analysis of fat in fish meal was studied on 4 samples of meal, using 12 different solvents and extraction periods ranging from 4 to 32 h (13). All meal samples were dried in a vacuum oven 2 h at 80°C. Because fish oils are highly unsaturated and readily susceptible to oxidation, the fat becomes less soluble. According to the writer, the amount of ether-extractable fat can decrease by as much as 50% over a period of 9 months. The solvents studied were petroleum ether, hexane, heptane, ethyl ether, carbon disulfide, cyclohexane, benzene, methylene chloride, trichloroethylene, chloroform, acetone, and 1,4-dioxane. All solvents except acetone gave little increase in extraction value beyond 4 h. For acetone, 16–24 h was required. Ethyl ether, carbon disulfide, and cyclohexane gave quite similar values, which were slightly above petroleum ether, hexane, and heptane. In general, all solvents tested gave definitely lower extract values after the meal had oxidized.

In a 1942 summary report (14), the 2 most used solvents for extracting fat from fish meal were petroleum benzene and ethyl ether. Both had limitations as efficient solvents. Preliminary drying of the sample was more apt to decrease than increase the accuracy of the fat determination. None of the solvents or solvent mixtures in the procedures investigated gave accurate measures of the fat content.

In later studies, initial fat recoveries were much higher in fish meal when acetone was substituted for ether. Hydrolysis of the extracted meal with acid followed by a second extraction usually increased the yield of fat by about 1%. The sum of the 2 extracts represented total fat. This procedure (15) was adopted for determining fat in fish meal.

Collaborative study on 6 varied samples (16) was done to compare the present official 16 h crude fat extraction method with the 4 h high-heat Goldfisch fat extractor for feedingstuffs. The results by the latter method were consistently lower than those for the official 16 h method. The Goldfisch method was not recommended but could be used where time was an important factor.

Until the Goldfisch apparatus was introduced, crude fat in feeds was extracted 16 h at low heat by the Soxhlet extractor. By 1950, most of the state and federal laboratories were using the 4 h high-heat extraction of the Goldfisch apparatus. Because of so much interest in the shorter method, which gave only slightly lower results than the 16 h extraction, a second collaborative study was done (17). The method was changed to read: "Use thimble with porosity permitting rapid passage of the ether. Extraction period may vary from 4 h at condensation rate of 5–6 drops/s to 16 h at 2–3 drops/s."

In 1942, a report was made on fat in cooked animal foods containing cereals (18). The acid hydrolysis method gave more fat for dry dog food samples than did the direct ether

extraction. Agreement was close on canned dog foods. In a later study (19), it was shown that the acid hydrolysis method for fat always gave higher results than did the ether extract method, even for ingredients of canned dog food. It was surmised that the acid hydrolysate contained materials other than fat. A method was devised to determine true fat content by determining the free and total fatty acids and glycerol content of the extracts. Both canned and baked dog foods were analyzed. The results showed that the ether extract was almost 99% true fat, and the acid hydrolysate contained materials other than fat. Because of this study, the acid hydrolysis method was discontinued.

About 10 years later, a new acid hydrolysis method for fat in baked dog food was collaboratively studied and adopted official (20). After acid hydrolysis in a Mojonnier tube, a series of extractions were made that used alcohol, ether, and petroleum ether. The ether was finally evaporated and dried, and fat was weighed. Ten years later (1961) this method was recommended to include expanded dog foods and in 1976 to include intermediate moisture pet foods.

When urea is present, fat extraction by ethyl ether gives a positive bias because urea is slightly soluble in ether. To correct for this, the sample is extracted with water and dried before ether extraction.

Crude Fiber

In 1887, the first reported crude fiber analyses were done on hay, bran, and cottonseed meal, using the Weende method. Basically, the method is the same today: Extract the fat from 2 g, add 200 mL boiling 1.25% sulfuric acid, boil 30 min, filter, add 200 mL 1.25% sodium hydroxide, boil 30 min, filter through a Gooch crucible, wash with water, alcohol, and ether, dry, weigh, ash, cool, and weigh. Calculate the crude fiber content from the sample weight loss.

Until the turn of the century, the main concern of the crude fiber method was keeping the acid and alkali at 1.25% by titration, keeping the 200 mL volume constant by using condensers over beakers, and assuring that the crude fiber did not contain appreciable quantities of nitrogenous bodies. After the introduction of the pentosan determination, interest developed for a pentosan-free crude fiber method. Konig (21) introduced a practically pentosan-free crude fiber method which consisted of boiling the material with glycerol containing sulfuric acid (boiling point 131–133°C).

The first collaborative study comparing the official method with the Konig fiber method showed that the latter, although practically pentosan-free, did not completely remove the albuminoid matter from materials high in protein. The Konig method was modified: Following the glycerol-sulfuric acid treatment, transfer the residue to a flask, add 200 mL hot 1.25% sodium hydroxide, boil 30 min, filter through a Gooch crucible, dry, weigh, and ash. The loss of weight equals the amount of crude fiber.

The modified Konig procedure gave fiber almost free of pentosans and albuminoids but the high boiling temperature caused a hydrolytic action on the cellulose. Testing the official and Konig methods on pure cellulose showed twice as much loss with the former. Because the cellulose loss by the Konig method was much larger than the error caused by the presence of pentosans in the present official method, the Konig method was abandoned.

Another fiber method was proposed: Instead of filtering the acid solution at the end of the first digestion, add 200 mL 3.52% sodium hydroxide, boil 30 min, filter through linen, wash with hot water, wash with 1.25% boiling acid, wash with hot water, transfer to a Gooch crucible, wash with water, alcohol, and ether, dry, weigh, ash, cool, weigh, and calculate crude fiber. No time was saved because the filtration was slowed by reprecipitation of solids, which also caused a positive bias.

Since the official method is an empirical one, any deviation from it will give different results.

The details of the crude fiber method (22) were studied to demonstrate that small changes probably cause problems within the bounds of the official method and to determine what details must be included in a proposed method to make it more satisfactory. Condensers, filtration delays, containers, ether extract removal, degree of fineness of grind, filtering media, and effect of asbestos were considered.

The conclusions were as follows: Follow directions of the method carefully. Check 1.25% acid and alkali by titration. Use especially prepared asbestos. Use Liebig water-jacketed condenser. Keep solution in container at 1–1½ in. deep. Use butchers' linen, 4.5 threads/in.

The modified method was as follows: Extract 2 g dry material with ether. Transfer the residue and 0.5 g asbestos to the digestion flask. Add 200 mL boiling 1.25% sulfuric acid, connect to condenser, heat to boil in 1 min, and boil exactly 30 min. Rotate flask every 5 min. Immediately filter through linen in a fluted funnel and wash with boiling water. Wash the residue back into the flask with 200 mL boiling sodium hydroxide by a blow tube. Allow ca 3 min between flasks returned to the heater. Boil solution exactly 30 min and filter immediately through a Gooch crucible prepared with a thin layer of ignited asbestos. Wash with boiling water and about 15 mL alcohol. Dry at 110°C to constant weight, cool, weigh, and incinerate. Cool in desiccator and weigh. Report the weight loss as amount crude fiber.

After minor changes and collaborative studies done in 1921 and 1923, the method was adopted official.

Many papers written later stressed that many analysts did not follow the method carefully. One important step was to rotate the beaker every 5 min to bring the fiber lodged on the walls of the beaker back into the solution. Because each sample would be swirled 5 times in both acid and alkali solutions, a total of 120 times for 12 samples would be required. In 1945, our laboratory was using Labconco apparatus. To swirl the beaker required one hand on the digestion beaker and the other on a twist knob to lower the heater from the condenser. The beaker was then latched back in place. To accomplish this task would take too much of a technician's time. Many control laboratories around the country using this same apparatus were probably not following the method carefully on this important point. To better follow the method, our laboratory mounted the extractors on heavy duty springs, which allowed the analyst to shake the entire 6-unit extractor with one hand in 1–2 s.

After the 1957 AOAC meeting, the AOAC–AOCS Crude Fiber Liaison Committee was organized. Four years later the committee presented a completely revised crude fiber method, which was adopted official final action (23). The main points are listed:

1. Filtering device using a 200 mesh stainless steel screen
2. Porcelain crucible instead of a filtering crucible to ash the residue
3. Asbestos, especially treated
4. Antifoam agents
5. Liquid preheater
6. Beaker rotated periodically

Probably the most important change was the California-type buchner filter funnel. The fiber method has remained the same to date.

Throughout the 1960s, many papers were published about acid detergent fiber and lignin. The AOAC fiber method was considered to have many incorrectable factors in conflict with the animal digestive system. One particular flaw of the method was the measure of lignin. Van Soest of Cornell University has published many studies on the acid detergent fiber method. In 1973, after collaborative study, a method was adopted official first action (24).

Ash

In 1887, the method for ash in cattle feeds was as follows: Char the substance at a low, red heat, exhaust the charred mass with water, burn the insoluble residue, add the ash to the residue from the evaporation of the aqueous extract obtained above, dry the whole at 110°C, and weigh. Determine carbonic acid and insoluble matter (sand and charcoal) in the product for the estimation of the pure ash.

In 1939, it was found that mixed feeds containing added oyster shells or limestone gave irregular ash results (25). A collaborative study was done on 3 samples: one without $CaCO_3$, one with 2% added, and one with 4% added. Each sample was ashed at 550, 600, 650, and 700°C for 2, 5, and 16 h. Results showed that the ashing temperature should be at least 650°C for at least 2 h.

The official method for ash is as follows: Weigh 2 g sample into a porcelain crucible and place in a preheated furnace 2 h at 650°C. Desiccate, cool, and weigh immediately. In a later report, a 600°C temperature was agreed on because of potassium losses at the higher ashing temperature, even though some carbon may remain at the lower temperature.

Moisture

The 1888 method for hygroscopic moisture in cattle feeds was as follows: "Dry 2 to 3 g at 100°C to constant weight." By 1925, it was changed to "Dry 5 h under a pressure not to exceed 100 mm of mercury." Other methods are toluene distillation, drying without heat over sulfuric acid in a vacuum desiccator, drying in air oven 2 h at 135°C, and the Karl Fischer titration. The new Karl Fischer titrators with electrometric end point detection, automatic dispensing of KF reagent, and coulometric generation of iodine have made this instrument very attractive.

Minerals

The first feeds containing the minerals calcium, phosphorus, iodine, and salt came to the attention of AOAC in the middle 1920s. The calcium method is as follows: Ash the material, dissolve the residue in hydrochloric acid, dilute to volume, take an aliquot, adjust the pH, bring to a boil, add ammonium oxalate, and let stand overnight. Filter, transfer the precipitate to a beaker, add sulfuric acid, bring to a boil, and titrate to a slight pink with standard potassium permanganate. Calculate the percent calcium. The calcium method was collaboratively studied and tentatively adopted (26). Later the pH was changed from 3–4 to 2.5–3 to prevent co-precipitation of magnesium oxalate. After a successful collaborative study, the method was adopted official first action.

Phosphorus is determined from the same sample preparation by using the fertilizer gravimetric quinoline molybdate procedure (27).

Calcium and phosphorus along with nitrogen have also been analyzed simultaneously on an automated system (28). The sample is first digested on a block digester. Phosphorus and calcium are determined by measuring the absorbances of the molybdovanadophosphate and calcium-cresolphthalein complexes at 420 and 570 nm, respectively. Routine testing for calcium in ration feeds is now done by atomic absorption spectrophotometry.

The iodine method is as follows: Extract the mineral with alcohol, acidify the extract, oxidize with hydrogen peroxide, extract with carbon disulfide, and compare the solution with a similarly prepared standard. This method was replaced with the Knapheide-Lamb method (29) which appeared in *Official Methods of Analysis* (1935) 4th Ed. and was long and tedious. A method by Elmslie-Caldwell was shorter and simpler than the Knapheide-Lamb method, and preferred for routine work (30). A collaborative report showed the mean averages of results by the 2 methods were the same. By 1950, both were official methods.

A screening procedure was proposed for the determination of iodide in feeds and plants by ion-selective electrode analysis (31). It compared favorably with the AOAC methods for feeds containing only the iodide ion.

In 1940, AOAC had no procedure for quantitative analysis of salt in feeds. The usual way to measure salt was to determine the chlorine content and calculate the percentage of sodium chloride. Chlorine from sources other than salt was often present. If total chlorine was determined, the organic matter had to be destroyed by ignition or digestion. In either case, problems arose with loss of chlorine or incomplete digestion. Most of these methods were tedious.

Because the total chlorine content of the ingredients is not available, it is better to measure the water-soluble chlorine. A method was developed (32) to extract water-soluble chlorine from feedingstuffs with dilute ferric sulfate solution followed by addition of dilute ammonium hydroxide. This procedure precipitated the protein to give a clarified solution in a short time. Solutions were added to the sample by pipet to make a definite volume, samples were shaken and filtered, and an aliquot was taken for titration with $AgNO_3$ and KSCN. After a collaborative study, this was made the official method for soluble chlorine in feedingstuffs.

In 1969, a collaborative study on a potentiometric titration method for soluble chloride in feeds (33) was conducted, recommended, and approved for official first action.

A need for determining manganese in feeds arose when a deficiency of this element caused perosis in chicks. Collaborative studies were done on a colorimetric potassium metaperiodate method, which involved dry-ashing, nitric–sulfuric acid solution digestion, evaporation, phosphoric acid digestion, filtration, oxidation, and reading of color (34). This acid-soluble manganese method was adopted official.

The first method for copper in feeds involved development of a color complex with ammonia and tetraethylene-pentamine. Later, a collaborative study of copper in feeds by atomic absorption spectrophotometry (AAS) (35) was successful, and the method was adopted official first action. In fact, the older colorimetric procedures for minor elements in feeds have generally been replaced by atomic absorption spectrophotometric procedures, which are faster, more specific, and usually more sensitive. A giant step forward on the speed and accuracy of the AAS procedure came with the development of electrobalances, which can be interfaced to a microprocessor or computer. All data from sample weight, dilutions by weight, and calculations can be stored and printed accurately and quickly. There is really no comparison between this technique and the gravimetric, colorimetric, or titrimetric procedures of a decade or more ago.

Sampling and Sample Preparation

Feed sampling and sample preparation are important parts of feed analysis Up to this period, information on what tools

to use and how to use them has been limited. A 1940 report on sampling feedingstuffs (36) compared 3 triers: (A) Wheat trier, 11½ in. open end, tapered; (B) flax trier, 18½ in., 10¼ × 7/16 in. slot on tapered tube; and (C) single tube, slotted trier, length over 31 in., diameter ¾ in., and 24 × 9/16 in. slot. Samples were taken from bagged protein concentrates and alfalfa products by each trier and analyzed for protein and ash. The conclusion was that the type of trier is of no significance.

A later study was made using the same triers plus 2 longer double tubes, one ⅝ in. diameter and 9 slots, the other ⅞ in. diameter and 6 slots. The conclusion was much the same as that of the previous year.

Nothing appeared in *Official Methods of Analysis* on sampling grain and stock feeds until the sixth edition, 1945. The official first action procedure was to take cores from not less than 10% of bags present but not more than 20 bags. Samples were to be thoroughly mixed on oilcloth and reduced by quartering.

In 1955, a report on sampling compared probe devices with hand sampling of pellet-ground feed mixtures. The samplers were 36 in. long, slotted cone tip: (1) ¾ in. diameter with ½ × 24 in. slot; (2) 1¼ in. diameter with ⅞ × 26 in. slot; (3) 1½ in. diameter with slot 4 opening 1¼ × 2 in. along one side. Hand samples were also taken. The conclusions were that the smallest probe did not operate consistently well with this type of mixture, sampler 3 showed improvement over the other types, and hand methods were the most satisfactory.

Questionnaires were mailed to all state laboratories asking for exact dimensions and descriptions of triers they used, how they were used, and how many cores were taken. On the basis of this information, the single tube trier was the most satisfactory: single slot, 28–30 in. overall length, ¾ in. diameter, and a 20–24 in. slot.

As a result of a survey on feed sampling techniques and the Miles and Quackenbush report (37), the Associate Referee on feed sampling made a significant recommendation (38) to change the sampling procedure as follows: "From lots of 1–10 bags, sample all bags; from lots of 11 or more, sample 10 bags." This was adopted first action.

A study on sampling bulk commercial feeds (39) reported little difference between analysis of 20 cores and 10 cores. Therefore, it was recommended that the official sampling procedure be amended to specify that at least 10 cores from different regions be collected when bulk feeds are sampled.

After a 700–1000 g, 10-core composite dry feed sample arrives in the laboratory, good sample preparation for chemical analysis is very important. A sample can be ruined if improperly prepared. Minimizing possible errors and biases requires use of a well made gated-riffle, which equally divides the sample before and after grinding. Also required is an efficient, relatively high volume, easy to clean mill, such as the Brinkman mill, with a 0.75 mm sieve or the equivalent to produce a finely ground sample of most dry feed materials. The sample is mixed before dividing, ground, and mixed after grinding. Riffling the sample 2 or 3 times yields approximately 50 g for chemical analysis.

Feed Adulteration

The study of feed adulteration was first started by the Association about 1914. The apparatus used to examine confined feeds included a set of 20–70 mesh sieves, a hand lens, a compound microscope with low and high power lenses, dissection needles, reagents for clearing and stains, and apparatus for making permanent slide mounts.

Types of adulteration found in these early days included total substitution of one material for another, e.g., corn feed meal for hominy feed; use of inferior quality substance, e.g., linseed meal and flaxseed screenings; addition of coca shells to compounded feed; accidental addition; omission of an expensive ingredient; and intentional subtractions and additions.

Collaborative studies were conducted on such samples as wheat bran and screenings, linseed oil meal with cottonseed meal and hulls, scratch feed with grit and weed seeds, cottonseed meal with hulls, horse feed, corn bran in hominy feeds, oat hulls in oats, rice hulls in rice bran, and bone in meat meal. Analysts with training and experience could detect adulterated materials with a high degree of accuracy and could estimate the quantity fairly well.

In 1928, stock feed was adulterated with dried buttermilk. Adulteration could be determined by microscopically identifying the *Bacillus lactis* stained with methylene blue. In a modified procedure, a slide stained with Carbol-Fuchsin could detect as little as 0.1% adulteration with dried buttermilk.

Small quantities of ferrous salts, copper salts, and potassium iodide in feedingstuffs could be detected by the following techniques: For ferrous salts, sift the feed over the surface of a paper that has been moistened with potassium ferricyanide solution. After a few moments, wash off the feed with water. If ferrous salts are present, blue spots will appear. For copper salts, use ammonia carbonate in ammonium hydroxide solution instead of potassium ferricyanide. For iodide, use starch and bromine water instead of potassium iodide. The method is sensitive, and it was adopted.

Starch in condensed milk products could be detected by boiling a sample in water a few minutes, placing a few milliliters of cooled mixture in a test tube, and adding a drop of I-KI test solution. If starch was present, a blue color was produced.

In the early 1930s it had been shown that fluorine from rock phosphate used in feed was causing toxicity in animals. In 1943, a collaborative study on a fluoride method developed by Willard and Winter (40) specified alkaline fixation while ashing, steam distillation of the volatile hydrofluorosilicic acid to separate interferences of other elements, and titration of fluoride with thorium nitrate in the presence of an indicator to form a pink lake (41). Fluoride bleaches the pink lake, and the degree of bleaching is a measure of the fluoride content.

The official AOAC method mentioned above is long and tedious(41). When the selective fluoride electrode (42) was introduced, a new tool for measuring fluoride in feeds was then available to analysts. A collaborative study of an ion-selective electrode method for the direct determination of fluoride in feeds was conducted by the Associate Referee (43) in 1975. Because it specified direct dissolution of the sample in 1.0M HCl, it eliminated the ashing, steam distillation, and titration steps of the official method, not only saving considerable time but also possible sources of errors. Sodium citrate and sodium acetate produced the best buffer system to give more reproducible potential measurements than any other used. This was adopted official first action.

Microscopic examination of feeds dates back to the beginning of the century. It has been a very useful tool to analyze both qualitatively and quantitatively the adulterants in feeds. An experienced microscopist can quickly analyze the quality of a feed in a way the analytical chemist cannot. Industry and control laboratories have recognized how helpful the microscopic analysis really is.

In 1953, at Lexington, KY, the American Association of Feed Microscopy was founded. It adopted a constitution,

collaborated with the Association of American Feed Control Officials on definition of feedingstuffs, developed techniques, set up a collaborative check sample program, published a manual, and was recognized by AOAC. Three method areas were established: identification of vegetable tissues; identification of animal tissues; and identification of major mineral constitutents. A very good historical report on feed microscopy was published in 1963 (44). A collaborative study on the basic procedure for feed microscopy was done in 1964, recommended, and adopted by AOAC (45).

Sugars

The official first action method for sugars in feedingstuffs appeared in the 3rd edition of *Official Methods of Analysis* (1930). Briefly, the sample was boiled in 50% alcohol, filtered, clarified with lead acetate and sodium carbonate, filtered, and diluted to volume. An aliquot was taken to determine the reducing sugars. If sucrose was to be determined, an aliquot was hydrolyzed with HCl, neutralized, and diluted to volume. An aliquot of this was taken to determine the reducing sugars by the Munson and Walker general method (46). The solution aliquot was added to 25 mL each of $CuSO_4$ and alkaline tartrate, brought to boil in 4 min, and boiled exactly 2 min. The Cu_2O precipitate was filtered, washed, dried, and weighed. Sucrose was calculated by referring to the Munson-Walker Tables. The method was made official final action the next year, and has remained much the same with occasional modifications. For an analyst using the method for the first time to analyze total sugars in animal feeds, it was not simple. In 1974, a simple, precise, volumetric sugar method was developed from parts of several methods (47).

A method for total sugars as invert in animal feeds (48) was developed, collaboratively studied, recommended, and adopted official first action. The method involves hot water extraction of the sample, inversion of the sugar with hydrochloric acid, and a direct titration of a standard Soxhlet solution. The key points to a successful measurement of the sugar are as follows: Run a pretrial of the unknown solution to determine within 1 mL the final titration with the sample solution; bring to boil in 2 min and complete the titration in 1 min with a strong oblique light to facilitate seeing the color change at the end point.

Vitamins

In 1932, large quantities of fish oils, cod liver oil, and liver meal were used in poultry feeds to supply vitamin D. An Associate Referee proposed a method to measure the potency of vitamin D on the basis of differences of bone ash of the tibia of chickens fed a rickets-production ration for 5 weeks and those fed the same ration with materials to be assayed. The method was studied collaboratively and looked promising. Two years later, with some changes, it was tentatively adopted (49).

In the following years, various studies of cleaning, dissecting, fat extraction, composite ashing of the tibia, changes in feeding times, and reference standards led to rewriting the method. Further studies showed that bones could be stored in alcohol, ashing for 1 h at 850°C is adequate, group ashing is as reliable as individual ashing and saves time, and there is a linear relationship between bone ash and body weight for all levels of vitamin D. It was also determined that the middle toe of the chick could be used to replace the tibia for ashing. This sensitive procedure was much more rapid, and the chicks did not have to be killed.

Microbiological and fluorometric methods for determining vitamin B_2 or riboflavin have been collaboratively studied on dried milk, yeast, dehydrated alfalfa meal, whole wheat, and flour.

In 1951, a spectrophotometric method for vitamin A, using antimony trichloride, was adopted official action (50). A few years later, some of the manufacturers of vitamin A encapsulated it in a matrix called stabilized vitamin A, which was not extractable with hexane. Another problem arose when manufacturers added to feeds the antioxidant diphenylphenylenediamine, which caused interference in the vitamin A determination. Sampling caused problems because of the particle distribution of the high potency granules of stabilized vitamin A. These and other problems kept the method in first action status.

A new, shorter method (51) was reported for vitamin A in mixed feeds in 1958 and, after collaborative study, was recommended for first action adoption; the older method was deleted.

Carotene, pro-vitamin A, was first studied in 1937 (52). A tentative method, limited to hays and dried plants, was adopted in 1941. Two years later, a collaborative study showed that by using a MgO-packed column, carotene, cryptoxanthin, and impurities could be separated from the crude carotene solution. Collaborative studies of various modifications were made through the years. In 1970, a new method (53) for determining carotenes and xanthophylls in dried plant materials and mixed feed was collaboratively studied and adopted as the official method.

Drugs in Feeds

Sulfaquinoxaline, used in poultry feeds to control coccidiosis, was the first drug studied by AOAC. The method (54) was as follows: Treat the sample with 0.5N NaOH, take an aliquot, add $ZnSO_4$ solution to precipitate proteins, filter, take an aliquot, and acidify with 0.1N HCl. Add sodium nitrate, ammonium sulfamate, and the coupling reagent, *N*-(1-naphthyl)ethylenediamine dihydrochloride, to produce a purplish red color which is measured on the spectrophotometer. It is the arylamido group that is diazotized and measured by the Bratton-Marshall reaction (55).

Later, it was found that the above method did not always recover all of the drug added, especially from aged or pelletized feeds. A new method was devised which used stronger alkali, HCl instead of $ZnSO_4$, and careful observance of time.

If arsanilic acid is also present, the sulfaquinoxaline is removed from the clarified solution with ether, leaving arsanilic acid to be determined. Total absorbance minus that for arsanilic acid gives absorbance due to sulfaquinoxaline. The method was collaboratively studied and adopted (56).

Because of criticism that the official method did not recover guaranteed amounts of sulfaquinoxaline, a new method was developed (57). Briefly, sulfaquinoxaline is extracted with dimethyl sulfoxide and chromatographed through an Al_2O_3 column. The isolated sulfaquinoxaline is diazotized, coupled in presence of zirconium, extracted with butanol, and measured at 550 nm. Arsanilic acid remains in the final solution and can be measured.

Development, collaborative study, and adoption of methods for determining drugs in animal feeds has been one of the most active areas for AOAC in recent years. Two more sulfa drugs, nitrophenide, used to control coccidiosis in poultry, and enheptin, used for prevention of blackhead in turkeys, appeared in 1952. In 1954, a modified method for enheptin and a new method for *p*-arsanilic acid were collaboratively studied (58). Nitrofurazone, a coccidiostat in poultry feeds, was first collaboratively studied in 1956 (59). A colorimetric

determination of nitrofurazone and furazolidone in feeds and premixes (60), based on the reaction of phenylhydrazine hydrochloride to form bright red 5-nitrofurfural phenylhydrazones, was shown to be rapid, simple, accurate, and reproducible. The procedure was modified in a collaborative study of furazolidone and bifurans in feeds (61) by using an Al_2O_3–$Mg(OH)_2$ column to minimize interferences and eliminate the pre-extraction step.

A liquid chromatographic (LC) method was presented to determine furazolidone, nitrofurazone, ethopabate, and carbadox in medicated feeds (62). A colorimetric method was adopted for nicarbazin (63). LC methods are superior to colorimetric methods because of their simplicity and specificity.

The drug diethylstilbestrol first appeared in 1955; cattle feeds were supplemented at the 5 mg/lb level. A search for a method of analysis began in 1956, and a method was adopted in 1959 (64). In 1969, a gas chromatographic (GC) method (65) for determination of diethylstilbestrol in feeds was developed that used dienestrol diacetate as an internal standard. The extractant was 7% ethanol in chloroform. A volatile derivative of diethylstilbestrol was made using bis-(trimethylsilyl)acetamide. Recoveries averaged 100.6% with a 5% coefficient of variation for the 0.0011–0.0022% range. Another GC method was developed based on reaction with dichloroacetyl chloride to form an ester measurable with an electron affinity detector and with dioctyltetrachlorophthalate as internal standard.

A fluorometric method was developed to determine diethylstilbestrol in liquid and dry feeds at the ppb level, but the purification step to remove interferences was long. The GC methods have become the methods of choice and are capable of reaching the 2 ppb level.

Roxarsone is used in poultry and swine feed to increase weight gain. A colorimetric method (66), which is rapid and satisfactory for routine work, was developed. The color reaction is considered specific for this drug because the common feed medicants are adsorbed at this pH by activated charcoal. The drug is extracted with 2% sodium bicarbonate solution, and proteins are precipitated by pH adjustment; after centrifugation the yellow solution is treated with charcoal at pH 12, filtered, and read from one cell. A second cell is acidified and read. The difference in absorbance is due to roxarsone. Other techniques were based on the determination of arsenic in the molecule by a colorimetric procedure and by atomic absorption spectrophotometry with the arsenic electrodeless discharge lamp. A new colorimetric method (67) was later developed, collaboratively studied, and made official first action.

Piperazine, a wormer for swine and poultry, was first reported in 1957. A number of methods were proposed: steam distillation and a color reaction with quinone; formation of piperazine acetate with acetic anhydride, and weighing; and GC analysis of piperazine as the diacetylpiperazine. In 1967, a colorimetric method was developed (68) which was a water extraction of piperazine and subsequent reaction with hot alcoholic *p*-benzoquinone to form a colored complex. This method was collaboratively studied and adopted official first action.

The official spectrophotometric method for phenothiazine was adopted in 1959 (69). A method for steam distillation of nicotine followed by ultraviolet spectrophotometric measurement was adopted in 1964 (70). A method for nithiazide (71), based on DMF extraction, column chromatography, and color development in alkaline solution with procaine HCl and a coupling reagent, was developed, studied, and adopted official first action.

Reserpine is used in feeds at the 0.002% level for its tranquilizing effect, which also promotes growth. The first AOAC method, a colorimetric procedure, was satisfactory for most feeds but a more sensitive method was needed. A fluorescence procedure was developed, collaboratively studied, and adopted. It was further studied in 1962 at a 0.2 ppm level and again adopted (72).

Zoalene (3,5-dinitro-*o*-toluamide), a coccidiostat for poultry feeds at the 0.0063–0.0125% level, appeared on the market in 1960 (73). Also in 1960, the feed industry introduced ethoxyquin, an antioxidant to prevent feed aging; a photofluorometric method for ethoxyquin was adopted by AOAC (74). Amprolium, a new coccidiostat introduced in 1961, was first determined colorimetrically. A shorter and more sensitive method using fluorometry was developed and adopted (75). A Bratton-Marshall colorimetric method was adopted for acetyl-(*p*-nitrophenyl)sulfanilamide, a coccidiostat used in poultry (76).

Ronnel, a systemic insecticide used in cattle feeds to control grubs, flies, and other parasites, was marketed either as 40% or 5% concentrate. In 1962, a collaborative study was done on ronnel in mineral-base mixtures (1.1 to 4.0%). The method developed, which used a methanol extraction and read absorbancy at 4 different UV wavelengths, was then adopted (77). The following year (1963) a method on complete feed mixtures (0.04 to 0.3%) was developed, collaboratively studied, and adopted (78).

It was later found that the 2,4,5-trichlorophenol decomposition product and ronnel can be separated on a column of Dowex 2-X8 ion-exchange resin. Trichlorophenol remains on the column and ronnel can be measured in the effluent. A collaborative study on this modified method was successful, and the method was adopted; the earlier method was deleted (79).

In 1967, further studies were made on ronnel, using acetone extraction, Florisil cleanup of the extracts, and electron capture gas chromatography for measurement. A GC flame ionization detector method for ronnel in cattle feeds at levels of 0.038–0.055% was studied in 1969 (80). This method involved extraction with acetone and direct chromatographic measurement; it measured true ronnel and was made official first action. The 1965 method for ronnel was repealed.

A later report of a GC method (electron capture detector) proved that the low recoveries of ronnel on samples stored for long periods of time actually were caused by partial degradation of ronnel to 2,4,5-trichlorophenol.

A *p*-aminobenzoic acid method was developed using the Bratton-Marshall reaction (81). Ethopabate (methyl-4-acetamido-2-ethoxybenzoate) is a coccidiostat used in combination with amprolium in poultry feeds. The first method (1964) involved diazotization and coupling of the free amine. The method (82) was slightly revised in 1965 and adopted. An LC method developed in 1977 was sensitive to 1 ng (83). A GC method for feed premixes at 0.8–8.0% level was developed in 1980 (84). A method was developed for dimetridazole based on extraction with hot methanol, chromatography on alumina, and absorbance at 318 nm (85).

Thiabendazole is an anthelmintic used in cattle and swine feeds. Over a period of several years, various methods were tried. In 1966, a method (86) that was applicable to all feeds containing 0.005–8.07% thiabendazole was developed, collaboratively studied, and adopted. The method involves a series of extractions into HCl, chloroform, and HCl, followed by reaction with zinc, *p*-phenylenediamine, and ferric ion to form a thiazine dye, which is extracted into butanol and measured.

Sulfamethazine is a medicant used in swine feeds, often with procaine penicillin. The method developed presented problems with colored extracts and incomplete removal of penicillin. In 1968, a modified method (87) was developed which removed the penicillin; it was adopted official first action. The LC technique is much preferred in the analysis of sulfonamide-medicated feeds because of its specificity and speed (88).

Additional methods have been adopted for nitrodan, an anthelmintic used in dog, cat, and poultry feeds (89); buquinolate, a coccidiostat used at the 0.00825% level (90); decoquinate (91), based on its fluorescence in alcoholic solution in the presence of calcium, magnesium, or strontium; nequinate, a coccidiostat used in poultry feeds at the 20 μg/g level (92); ronidazole, used to control blackhead in turkeys (93); aklomide, a coccidiostat (94); ipronidazole, used for control of blackhead in chickens and turkeys (95); sulfadimethoxine, a coccidiostat used in combination with ormetoprim (96); nifursol, used to prevent blackhead in turkeys and chickens (97); and pyrantel tartrate, an anthelmintic agent used in swine feeds at the 0.0881–0.0106% level. The method (98) developed for pyrantel tartrate was not applicable to feeds co-medicated with tylosin. In the LC method (99), the standard additions technique is used to compensate for the feed matrix effect.

Lasalocid is an antibiotic coccidiostat for poultry. A spectrophotometric method (100) was developed, collaborated, and adopted. A microbiological assay method was also collaborated and adopted (101). An LC method for lasalocid in premixes (102) was reported to compare favorably with the biological method.

Carbadox is an antibacterial agent used in swine feeds. A colorimetric method (103) was developed which involved extraction with chloroform–methanol, a series of solvent-solvent extractions, and a reaction with stannous chloride to form a colored complex. The standard additions technique is used to compensate for the feed matrix effect.

An LC method (104) for carbadox, furans, and ethopabate seems to be superior to the official method because the extraction and cleanup are easier, several drugs can be determined at the same time, and it compares well with the colorimetric methods.

Microbiological Antibiotic Assays

The use of antibiotics as growth factors in animal nutrition was first reported in 1946, when it was shown that diets containing sulfasuxidine and streptomycin stimulated the growth of chicks (105). This attracted little attention until 1950, when it was reported that the fermentation products of *Streptomyces aureofacions* stimulated the growth of chicks on completely adequate diets which included B_{12} (106). These findings prompted the use of antibiotics in animal feeds.

In 1953, AOAC appointed William A. Randall as Associate Referee to study the assays of feed supplements and concentrates (107). In 1954, 3 large antibiotic producers, Chas. Pfizer, Commercial Solvents Corp., and Lederle Laboratories, a division of American Cyanamid, helped to set up the program. The assays from these companies, along with procedures developed at the U.S. Food and Drug Administration, were used for the initial test. The best aspects of each method were to be incorporated into one method which then would be submitted for collaborative study (108).

A collaborative study of penicillin, bacitracin, chlortetracycline, and oxytetracycline was started in 1955 and completed within a year. The methods were studied collaboratively and were then recommended for adoption as first action at the AOAC annual meeting in October 1956 (109).

From the time the first antibiotic methods were studied and approved to the present, many others have been developed. Now, there are official methods for bacitracin, chlortetracycline, erythromycin, hygromycin B, lasalocid, lincomycin, monensin, neomycin, novobiocin, nystatin, oleandomycin, oxytetracycline, penicillin, spectinomycin, streptomycin, and tylosin (110). Most of these antibiotics are assayed by either the plate diffusion method or the newer, turbidimetric assays.

In the plate diffusion method, antibiotics are extracted from the feed by use of different solvent types, such as acid–acetone, acid–methanol, and buffer–acetone. The extract is then diluted to a reference concentration. These assay solutions are plated on petri plates filled with one of several types of antibiotic media. The medium is inoculated with a specific bacteria for each antibiotic and poured into the petri plate. In the antibiotic assays, a standard curve is run along with the samples to be tested. The standard curve consists of 5 points of known concentrations with the midpoint serving as the reference concentration. The antibiotic inhibits the growth of bacteria in the agar. Results are obtained by measuring the zone of inhibition around the spot where the samples and/or standards are plated. The zone size is proportional to the concentration of the solution.

A newer technique, adopted in the last few years, is the turbidimetric assay. Samples are prepared and extracted as above, but test tubes are used instead of petri plates in this assay. The tubes are filled with an antibiotic broth medium and inoculated with a specific bacteria for the antibiotic to be tested. Samples and standards are then pipetted into the tubes, and the tubes are incubated 4–6 h. Growth is stopped at this point, and the tubes are read on a spectrophotometer. The turbidimetric method is a 1 day procedure, whereas the plate method takes 2 days.

The next phase in antibiotic assay development will incorporate LC methods. A few LC methods are now in use, e.g., for bacitracin (111).

Feed analysis activity in AOAC from 1887 to about 1925 was mainly concerned with protein, fat, fiber, moisture, and ash determination in straight products, such as corn, cottonseed meal, linseed oil meal, oats, tankage hay, wheat shorts, wheat middlings, and distillers' products. Then, tests for minerals were started. By 1940, vitamin methods were appearing in the journals. After 1950, methods for drugs in feeds were developed rapidly, reaching a peak about 1970.

Because of the development of new, powerful and automated apparatus using computers, the feed analyst will be able to test samples more quickly, with greater accuracy and specificity than before. This will be particularly welcomed by the drug analysts. With equipment such as the liquid chromatograph, the difficult and expensive analysis of certain amino acids may become routine tests in control laboratories in the near future.

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DRUGS IN FEEDS

Liquid Chromatographic Determination of Zoalene in Medicated Feeds

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A rapid, reliable separation and quantitation of zoalene (3,5-dinitro-*o*-toluamide) from feeds is accomplished by using reverse phase liquid chromatography (LC) and ultraviolet detection. An extraction technique which is similar to the present AOAC official colorimetric method is used before chromatographic analysis. This extraction is followed by an activated alumina cleanup and LC to separate zoalene from feed matrix. The methodology was applied to a variety of spiked feed matrices, and yielded good recoveries. Liquid chromatographic results were shown to correlate with colorimetric determinations.

Zoalene is a drug that is added to feeds intended for consumption by chickens and turkeys as an aid in development of active immunity to coccidiosis. Coccidiosis is an intestinal disease caused by a protozoan of the genus *Eimera*, and results in death. At least 15 species of *Eimera* have been isolated as detrimental to poultry. The use of an anticoccidial drug (coccidiostat) such as zoalene can control the parasite and minimize flock loss (1). Primarily, 2 levels of zoalene are used in feeds: starter levels containing between 0.0083 and 0.01875% zoalene, and grower levels containing between 0.004 and 0.0083% (2). The present analysis is an AOAC method that involves reaction with ethylenediamine followed by colorimetric determination (3).

The method described in this paper combines the established AOAC extraction and purification with liquid chromatographic (LC) determination. This technique easily and accurately resolved and measured levels as low as 40 ppm. The method offers specificity and freedom from interferences. These interferences may contribute to false increased levels if detected by the colorimetric method.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Waters Associates Model 6000A pump equipped with Model U6K septumless injector, Model 440 single wavelength absorbance detector with 254 nm wavelength filter (or equivalent), and μBondapak™ C_{18} column (10 μm). Omniscribe dual pen recorder (Houston Instrument) was used to monitor detection. Operating conditions: column temperature ambient; mobile phase of acetonitrile–water (30 + 70) with 1% acetic acid added to total volume; flow rate 1.5 mL/min (1300 psi); sensitivity 0.02 AUFS; wavelength 254 nm; chart speed 0.2 in./min. Under these conditions, zoalene elutes in 5 min.

(b) *Solvents.*—Add 850 mL LC grade acetonitrile (Waters Associates) to 150 mL deionized water. Filter and degas.

(c) *Activated alumina.*—Baker Purified, 100–200 mesh.

(d) *Standard stock solution.*—Accurately weigh 40 mg dried zoalene reference standard (Dow Chemicals, Agriculture Division, Organic Dept) into 1 L volumetric flask. Dilute to volume with acetonitrile–water (85 + 15). Stir to dissolve. This is equivalent to 0.004% zoalene (w/v or 40 μg/mL).

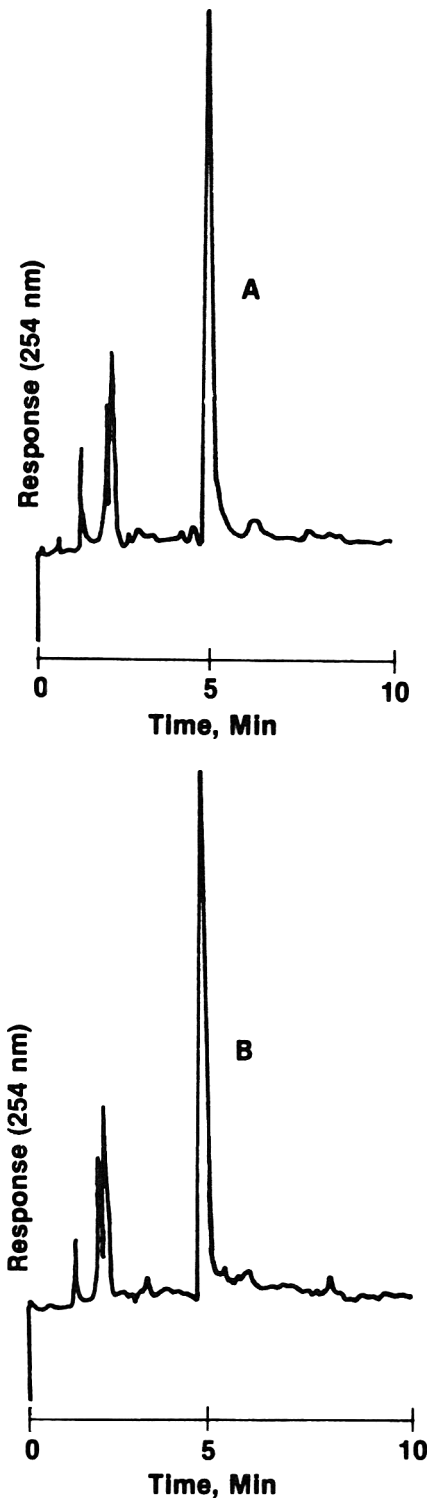

Figure 1. Liquid chromatograms of unextracted (A) vs extracted (B) zoalene standard.

Extraction and Purification

Extraction and purification methods are similar to those used in AOAC method (3): Accurately weigh 10 g ground

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Table 1. LC results (%) for recovery of zoalene from spiked feed samples

Feed	LC result	Spike	Expected	Actual	Spike rec.
1	0.0129	0.004	0.0169	0.0161	95.21
2	0.0074	0.004	0.0114	0.0106	93.00
3	0.0083	0.004	0.0123	0.01205	97.99
4	0.00536	0.004	0.00936	0.00927	99.00
5	0.00504	0.002	0.00704	0.0066	94.81
6	0.0082	0.002	0.0102	0.0099	97.13

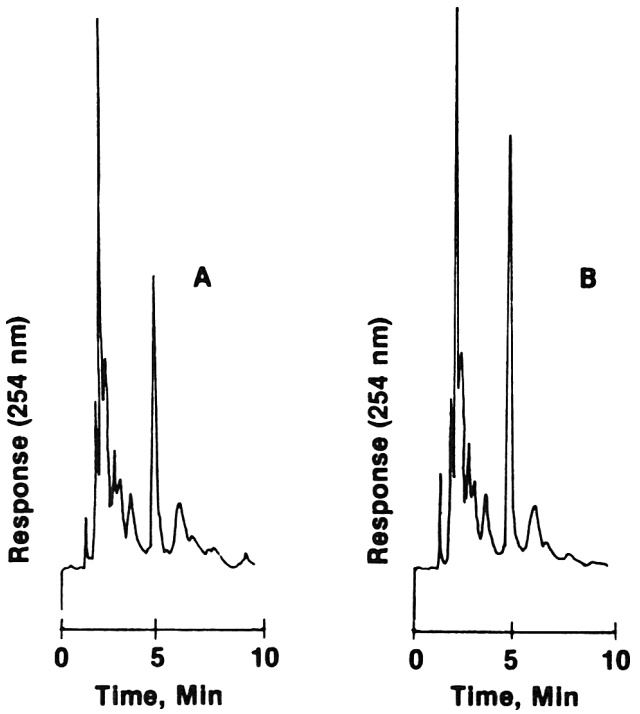

Figure 2. Liquid chromatograms of extracted medicated feed (A) and the same extracted feed spiked with 0.004% zoalene (B) before extraction.

Table 2. Determination of zoalene in feeds, using colorimetric and LC methods

Feed	LC	Colorimetric	Rel. diff., %
A	0.0137	0.0140	2.1
B	0.0078	0.0085	8.2
C	0.0080	0.0081	1.2
D	0.0055	0.0053	3.8
E	0.0050	0.0052	3.8
F	0.0082	0.0086	4.6
G	0.0137	0.0140	2.1

feed sample into 250 mL Erlenmeyer flask and add 65 mL acetonitrile–water (85 + 15). Warm sample on steam bath to 50 ± 5°C with occasional swirling and then let cool to room temperature. Add 20 g alumina and swirl ca 3 min. Filter with suction on medium porosity 40 mm diameter fritted glass funnel, transferring as many solids as possible. Break suction and wash remaining solids into funnel with minimum amount of acetonitrile–water (85 + 15). Filter with suction until dry. Break suction again and suspend solids in funnel with minimum volume of acetonitrile–water (85 + 15). Filter with suction until dry, break suction, and repeat wash, keeping total volume less than 100 mL. Quantitatively transfer combined filtrates to 100 mL flask and dilute to volume with acetonitrile–water (85 + 15). To prepare working standard, accurately add 10 mL standard stock solution (0.004% or 40 μg zoalene/mL) to 45 mL acetonitrile–water (85 + 15) in 250 mL Erlenmeyer flask. Heat, add alumina, and wash with acetonitrile–water (85 + 15) as directed for feed samples; this results in 100 mL final dilution volume (0.0004% or 4.0 μg zoalene/mL).

Determination

Filter samples and standards through 0.45 μm filters (Millipore Corp.) before injection. Use 10 μL injections, and dilute samples containing 0.01% zoalene with equal volumes of acetonitrile–water (85 + 15). Inject standard, inject sample, and repeat injection of standard. Measure peak height of standard *(PH')* and sample *(PH)* and determine zoalene with the formula:

$$\text{Zoalene, \%} = (PH'/PH) \times 0.004$$

Results and Discussion

To determine that no zoalene was lost during extraction and purification, the standard was injected before and after preparation. The average recovery for 7 analyses was 99.28% (range 95.7–102.47%). Figure 1 (B) shows a chromatogram which illustrates the recovery of a zoalene standard from the extraction procedure. Recovery was also high for feed samples (Figure 2 and Table 1). Six medicated feeds were extracted and analyzed by this LC method. These samples were then spiked with either 0.002 or 0.004% zoalene and again extracted and chromatographed. The recoveries ranged from 93 to 99% of the added drug with an average recovery of 96.19%, and demonstrate the linearity of results obtained by this procedure and its applicability to a variety of feed matrices.

Table 2 compares the AOAC colorimetric determination of zoalene and the LC method. Seven feeds were analyzed by both the colorimetric method and the LC method. Results from the 2 methods yielded a mean relative difference of 3.7%, suggesting good correlation.

The LC method described for the analysis of zoalene is straightforward, reliable, and accurate with good linearity of results over a wide variety of feeds and satisfactory correlation to the colorimetric method.

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ANTIBIOTICS

Microbiological Determination of Neomycin in Feeds and Formulated Products

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An AOAC modified method is described for the microbiological assay of neomycin, which has been adapted to include complete feeds, supplements, premixes, liquids, oil suspensions, boluses, and antibiotic-impregnated paper. The method features a more sensitive standard response line with a monolayer plating system. The use of a buffered plating medium in place of the water-prepared medium results in a curve with less degree of slope, which allows for more accurate interpretation of the standard response. The feed extract diluent used for standard response line dilution, which is prepared from exposure of the feed extract fluid to pH changes, heat, and sodium hypochlorite, has been eliminated. The constant salt concentration diluent used for the preparation of standards is the same as the salt concentration of the sample extract solution to be tested. Results for 50 commercial complete feeds and 50 commercial premixes received over the last 5 years produced an overall mean recovery of 101% with a mean percent recovery range of 80–112%. A statistical analysis of these 100 commercial, complete feeds and premixes, ranging in concentration from 47 g/ton to 70 g/lb, indicates the assay has little, if any, concentration-related bias. Precision and accuracy of the method was supported by laboratory studies of 20 assays that produced a mean recovery of 101% and standard deviation of 3.

The past decade has seen the introduction of many new product forms containing neomycin as a therapeutic agent for several animal species. Examples of these other than the previously marketed feeds are liquids, boluses, soluble powders, oil suspensions, and antibiotic-impregnated paper used in suspended caging for laboratory animal maintenance. The top layer of the paper is impregnated with neomycin to retard growth of ammonia-producing bacteria and thereby reduce unpleasant odors (1).

Modifications of the AOAC method (2) were required to obtain flexibility for such a diversity of products. It was necessary to eliminate the use of a feed extract diluent prepared from exposure of a feed extract fluid to acid, sodium hypochlorite (NaOCl), and heat. A constant salt concentration diluent applicable to the sample extract and as standard diluent for all the product forms has been substituted. In addition, a buffered plating medium produces a less steep, more sensitive response; this allows smaller sample size with greater dilution and, therefore, less interfering material from sample extracts. A monolayer plating system contributes to greater sensitivity, less variable zones of inhibition, and more expedient conduct of the assay. Comparison testing with the AOAC method to obtain sensitivity less than the 0.5 μg/mL lower level of the standard response only produced distorted, nonlinear zones of inhibition.

METHOD

Apparatus

See **42.198(a), (b), (c)** (2), plus the following:

(a) *Shaker, mechanical.*—NBS gyrotory or equivalent.

(b) *Tissue grinder.*—10 mL, 10–12 mm.

(c) *Stirrer.*—Variable speed, Fisher Scientific, Hamilton Beach or equivalent.

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Reagents

See **42.197(n)**, **(o)** (2), and **42.193(p)** (3).

Standard Solutions

See **42.256(a)** (2).

Standard response line.—Dilute aliquots of stock solution **(a)** with diluent prepared by diluting extractant, **42.193(p)** (3) (1 part) with 0.05M tris buffer (11.5 parts), to obtain 0.2, 0.4, 0.8, 1.6, and 3.2 μg neomycin base/mL. Reference concentration is 0.8 μg/mL.

Monolayer Plate

For assay, add appropriate amount of organism (usually 0.5%) as in **42.257(b)** (2) from cell suspension, **42.199(f)** (2) to agar medium No. 11, Bacto neomycin assay agar previously prepared, autoclaved, and cooled to 48°C. Distribute agar to plates in 8–9 mL monolayer. Incubate 16–18 h at 35–37°C.

Assay Solutions

(a) *Sample preparation for complete feeds (70–140 g/ton of neomycin base).*—Accurately weigh 7–14 g sample into 250 mL centrifuge bottle, add 100 mL 20% NaCl and 0.294% $CaCl_2$ in water (extractant) and shake 20 min on mechanical shaker. Centrifuge 3–5 min at ca 2500 rpm. Transfer 2 mL portion of supernate to 25 mL volumetric flask and dilute to volume with 0.05M tris buffer pH 8. (For example, a 7 g sample of 140 g/ton feed produces theoretical test concentration of 0.86 μg/mL of neomycin base and, likewise, a 14 g sample of a 70 g/ton feed.)

(b) *Sample preparation for high level feeds, premixes, and soluble powders (≥ 700 g/ton of neomycin base).*—Accurately weigh 1–4 g sample into 250 mL centrifuge bottle, add 100 mL extractant, and shake 20 min on mechanical shaker. Transfer 2 mL aliquot of supernate to 25 mL volumetric flask and dilute to volume with 0.05M tris buffer pH 8. Dilute 1.0 mL aliquot in extract solution (1 part) with tris (11.5 parts) diluent to ca 0.8 μg neomycin base/mL.

(c) *Sample preparation for boluses.*—Pulverize sample with mortar and pestle; proceed as in **(b)**.

(d) *Sample preparation for fluid products.*—Accurately weigh sample into tared centrifuge bottle and add extractant adjusted for sample size used to obtain a final extract volume of 100 mL. Proceed as in **(b)**.

(e) *Sample preparation for neomycin-impregnated paper (≥ 20 ppm).*—Accurately weigh 1–2 g previously cut and prepared sample. Transfer to 10 mL glass tissue grinder and add 3 mL extractant. Let sample soak 0.5 h. Grind sample to suspended pulp by rotating and manipulating tissue grinder plunger at medium speed for 2 min. Let sample settle 15 min. Dilute 1 mL aliquot with 11.5 mL tris buffer pH 8 (e.g., a 1 g sample of a 20 ppm preparation would have a theoretical concentration of 0.53 μg/mL of neomycin base/mL).

Experimental

(A) A typical commercial complete feed (S-810) for growing pigs was fortified with a 70 g/lb premix to a level of 70 g/ton.

Table 1. Effect of salt concentration on neomycin recovery

Sample	Diluent salt, %	Recovery, %	Mean recovery, %	SD
1a	1.6	99		
1b	1.6	99		
1c	1.6	100		
1d	1.6	100	99.4[a]	0.0033
2a	1.66	101		
2b	1.66	99		
2c	1.66	98		
2d	1.66	101	99.8	0.0175

[a]Probability of chance difference between 99.4 and 99.8 is 0.70, based on a 2-tail *t*-test.

The mixture was tumbled for 2 h to ensure a homogeneous mixture and then ground through a Wiley mill to pass a 1 mm screen.

(B) A study of a similar swine feed (S-581) was done with neomycin in tris buffer added directly to the sample at a fortified level of 70 g/ton.

(C) A comparative study of a cattle feed (B-291) and a swine feed (S-810) was done to illustrate the influence of 2-fold sample size differences on percent recovery.

Ingredient	*Percent*
S-810 (Swine)	
Ground corn	78.00
Soybean meal	19.0
Dyna K	0.1
Swine pre-mix 5S	0.25
Dicalcium phosphate	1.5
Calcium	0.75
Salt	0.4
	100.0
S-581 (Swine)	
Ground corn	77.35
Soybean meal	19.0
Salt	0.5
Defluorinate phosphate	1.9
Fat, solubilized	1.0
Vitamins and minerals	0.2
Selenium	0.05
	100.0
B-291 (Cattle)	
Shelled corn, coarse grind	89.03
Soybean oil meal, 49%	9.0
Limestone, ground	0.7
Salt	0.8
Vitamins and minerals	0.37
Sodium selenite	0.1
	100.0

Results and Discussion

Ten assays of the feed S-810 (A) produced a recovery range of 97–106% with a mean of 101% and SD of 3.3. The study of a similar swine feed S-581 (B) spiked at 70 g/ton, assayed on a single day using the same sample size for 10 assays, produced a mean recovery of 101% with a range of 98–104%. An SD of 2.7 also supports the precision and accuracy of the results. Effect of sample size was tested in both studies by using a 20 g sample, which is twice the size necessary and puts the theoretical results at the upper midpoint of the standard response line rather than at the center. This takes into consideration the impact of introducing more salt from the feed itself and its influence on results. In addition, the comparative study (C) of a cattle feed (B-291) and a swine feed (S-810) showed that normal differences in salt concentration within the feed sample itself apparently did not influence overall results. Results of 4 cattle feeds at 0.8% salt averaged 98% when a 20 g sample was used; when a 10 g sample was used for the same feed, results of 4 assays were 100%. Results of 4 swine feeds at 0.4% salt averaged 97% when a 10 g sample was assayed, and 99% when a 20 g sample was assayed for 4 feeds. These 16 assays had an overall mean of 98.8% and CV of 1.6. The antibiotic response of neomycin is increased in the presence of sodium chloride (NaCl) (4). This phenomenon can be readily demonstrated by keeping the antibiotic constant and varying the salt concentrations in the testing diluent. A buffer curve without salt will produce a much less sensitive response; therefore, it is imperative to equalize the salt concentration of both sample and standards when conducting the test. The amount of salt used in complete swine and cattle feeds ranges from 0.4 to 2.0% NaCl, and in a 20 g feed sample would alter the expected final salt concentration by a possible factorial range of 1.606–1.632 at the highest 2% level that might occur. To demonstrate salt concentration effects, a level markedly above the 1.632% level was tested. Four 14 g samples of a 70 g/ton feed were assayed at the normal 1.6% level vs four 14 g samples of the same feed extracted and diluted to levels of 1.66% (= 4% salt in a feed). All results were read on the normal 1.6% standard response line. The means were 99.4% and 98.8% for 1.6% and 1.66% salt, respectively. Probability of this small difference being due to chance was 0.70. Results of this study show that feeds containing up to 4% salt can be assayed with chance as probably the only factor influencing their difference (Table 1).

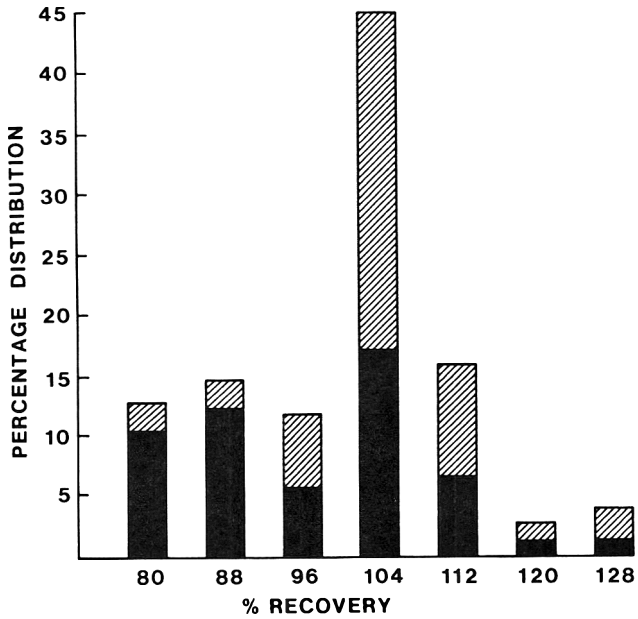

Figure 1. Histogram of % recovery of 50 complete feed samples (solid) and 50 premix samples (lined).

Results of 50 commercial complete feeds and 50 commercial premixes assayed when they were received over the last 5 years showed a mean percent recovery range of 80–112%, with an overall mean recovery of 101%. Figure 1 shows the distribution of percent recoveries for the 100 individual samples of complete feed and premixes. A weighted regression analysis, in Table 2, of amount food vs label amount showed

Table 2. Summary of 100 complete feeds and premixes received over 5-year period

Feed	Label	Mean recovery, %	No. of samples	Correlation (*r*)[a]	SD
Complete feeds (g/ton)	47.0	89	1		
	52.5	105	1		
	70.0	94	17		
	105.0	104	4		
	136.0	85	2		
	140.0	99	23		
	175.0	82	1		
	280.0	112	1	0.99	(0.114)
Premixes (g/lb)	0.227	111	3		
	0.245	98	2		
	0.875	97	1		
	0.980	107	1		
	1.4	106	1		
	2.1	95	1		
	3.5	80	1		
	4.0	104	1		
	5.25	84	2		
	14.0	107	20		
	20.0	106	5		
	35.0	104	1		
	70.0	107	7		
	227.0	106	4	1.00	(0.081)

[a]Correlation between mean amount found and amount on label.

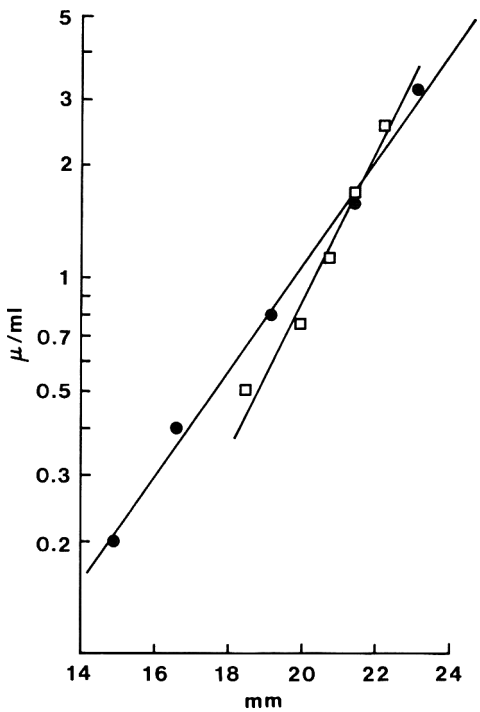

Figure 2. Comparison of slopes: AOAC standard response (□) and modified standard response (●).

regression coefficients well within the sampling limits of 1, which indicates the assay has little, if any, bias related to amount of neomycin in the feed. The correlations were calculated as the square root of the *r*-squares from the regression analysis. Samples received within the 5-year period of known problematic history unrelated to the assay were not included.

Slope comparisons of the AOAC standard response line and the standard response line from tris-buffered plating medium are shown in Figure 2. Use of pH 8 tris buffer for the plating medium, first reported by Williams and Wornick (5), is thought to resist in situ pH changes which may occur as a result of the organism, e.g., *Staphylococcus epidermidis*, fermenting glucose. This then would enhance the activity of neomycin, which is greater in the basic ranges, and may partially explain the 2.5 × greater sensitivity and less degree of slope obtained with the modified method. The increased sensitivity attained also allows for smaller assay samples, thereby reducing the amount of interfering material by increasing the sample extract dilution.

Extracting directly in centrifuge bottles on a gyrotory shaker rather than a wrist-action shaker eliminates a transfer step in the modified method. It was also empirically observed that more distortion and variation was present in the zones of inhibition from the 4 mL seed layer used in the AOAC method than in those from the monolayer modified system.

Summary

A 1.6% salt concentration diluent used for standard response solutions, prepared as specified, closely compares to that which will be present in the sample extraction fluid under test. Excess salt in complete feed formulations and larger sample sizes have been tested and the modified method has been shown to be statistically satisfactory. In addition, historical statistical data of commercial samples tested support the predictability of the experimental results presented. Technical modifications of the microbiological testing, while simplifying handling and decreasing expense of material involved, also increase sensitivity and versatility of the system. This modified method allows for testing a wide variety of product forms on the same standard response line, thereby promoting the capability and time-saving potential of the testing laboratory.

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EXTRANEOUS MATERIALS

Modified Flotation Technique for Quantitative Determination of Mite Populations in Feedstuffs

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Modifications are described to a previously published technique for quantitative determination of mite populations in animal feedingstuffs. In the modified method, the oil phase is kerosene instead of a mixture of kerosene and tetralin (1 + 1), and aqueous industrial methylated spirit is substituted for aqueous ethanol in the aqueous phase. Emulsion formation at the interface is considerably reduced by incorporating a pre-extraction stage. Examination of mites on the filter paper is made easier and quicker by staining them with Phloxin B. Trials carried out with known numbers of mites in samples of dairy feed concentrates indicate mean recoveries of 84–96% throughout the range tested. The results show that the accuracy of the technique is not affected by the level of infestation.

In a previous paper, a technique was described for the quantitative determination of mites in feedingstuffs (1). The method included the use of a specially designed flotation flask and a solvent mixture of kerosene and tetralin (1,2,3,4-tetrahydronaphthalene, $C_{10}H_{12}$) (1 + 1) to form an interface with 25% aqueous ethanol. Mites and insects were separated from the plant material by an overflow method. Although this technique was satisfactorily applied to a range of animal feedingstuffs, studies carried out in this laboratory and elsewhere indicated a number of limitations. These included the formation of an emulsion at the interface, which affected the repeatability of analyses. The use of kerosene and tetralin mixture as an oil phase was unsatisfactory because of its unpleasant odor. There was also a need to reduce costs and the time taken per extraction. This paper reports the effect of some procedural and apparatus modifications carried out to improve the original method.

METHOD

Apparatus

The extraction apparatus consists of a modified flotation flask made entirely of glass to the specifications given (Figures 1 and 2) (Jencons (Scientific) Ltd, Stanbridge Rd, Leighton Buzzard, Bedfordshire, UK), with its bottom sealed instead of housing rubber bung and stopcock. The flask can now be dismantled from the apparatus for emptying and washing. Valve A (Figure 2) positioned over the neck of the flask consists of a porous plastic disk (maximum pore size 90 μm), held in position by a plug of lead shot cemented together with resin-glue and then attached to the underside of the porous disk. The valve prevents the sample particles from settling in the neck of the flask and also ensures the oil is broken into small droplets before entering the flotation flask. The wire loop previously attached to the upper surface of valve A is no longer required. The apparatus also incorporates a modified paddle made from a rigid, right-angled plastic strip wedged into a slit made at the base of the shaft of the stirrer. The shape and dimensions of the paddle (inset Figure 2) are critical for optimum efficiency. The perforated PVC ring used in the previous method (1) has been simplified to a single 3 mm bore outlet E, positioned to aim a jet of oil at the junction of the shaft and paddle assembly. The outlet E is linked to reservoir D via 2-way stopcock F. A 3 piece Hartley funnel (9.5 cm id) is positioned below the gutter outlet of the flotation flask by a rubber bung in a 1 L filtration flask, which is attached to a vacuum source via a 500 mL filtration flask functioning as an oil trap.

Reagents

The oil phase consists of deodorized kerosene (s.g. = 0.786), and replaces the mixture of kerosene and tetralin (1 + 1). A 12.5% solution of industrial methylated spirits (IMS) in demineralized water (s.g. = 0.98) replaces 25% aqueous ethanol as an aqueous phase. The Phloxin B dye facilitates the detection and counting of mites on filter paper.

Pre-Extraction

Add 100 mL boiling demineralized water to pre-weighed amount of sample in 250 mL beaker. Add 30 mL methylene blue solution (0.3 g in 1 L IMS) and stir contents thoroughly to ensure adequate mixing. Wash down sides of beaker with 12.5% IMS. Place beaker in vacuum desiccator and reduce pressure for 20–30 s to remove any trapped or dissolved air from sample. Remove beaker from desiccator and place in water bath maintained at 70°C. Immediately add 3 mL freshly prepared Phloxin B solution (5 g in 500 mL 12.5% IMS) and 3 mL saturated solution of sodium chloride. Stir contents of beaker and wash down sides with 12.5% IMS. Set up apparatus as shown in Figure 2 with stirrer raised above flotation flask. Wet entire inside of flask with 12.5% IMS, using wash bottle, until 12.5% IMS fills neck of flask. Place valve A in neck of flask. After beaker containing sample has set in water bath 7 min, remove beaker and pour contents into flask. Thoroughly rinse beaker with 12.5% IMS to remove any particles adhering to sides and add rinse to flotation flask.

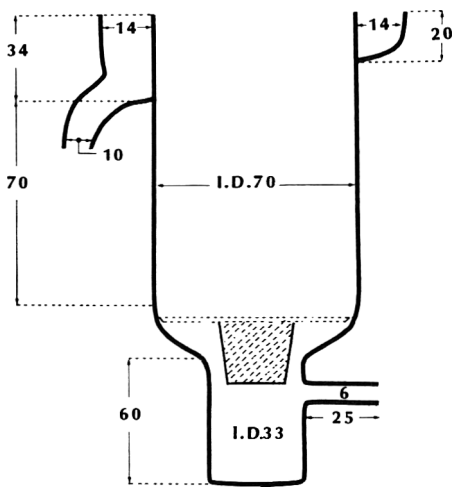

Figure 1. Diagram of flotation flask, showing dimensions in mm.

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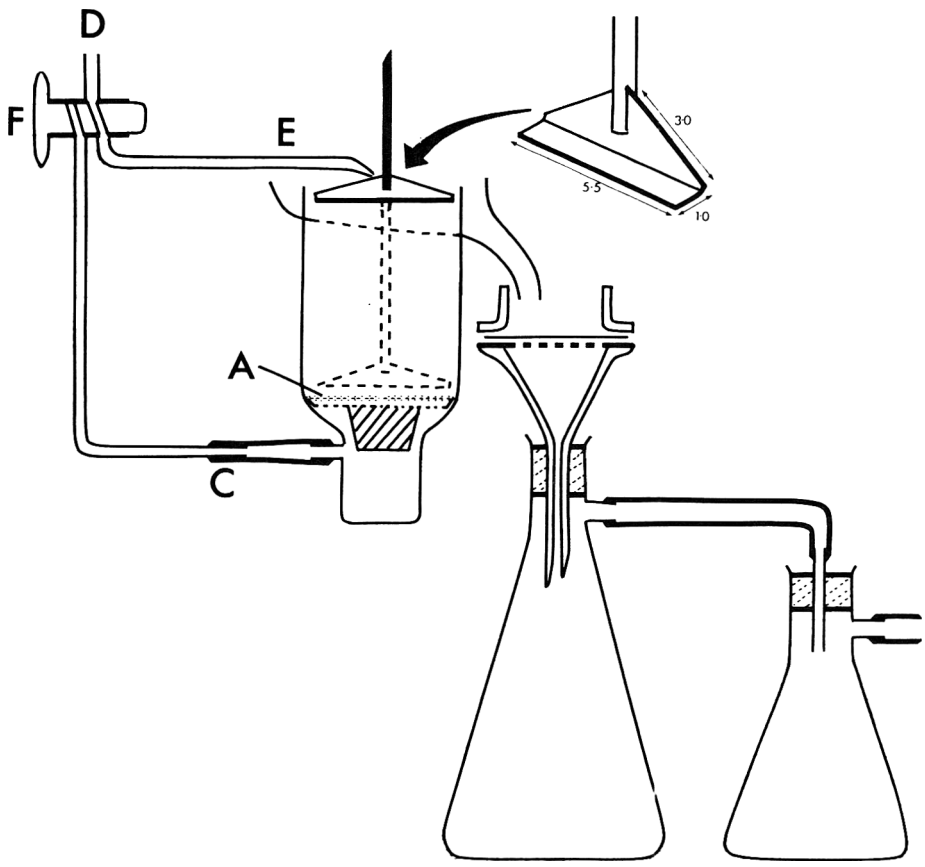

Figure 2. Diagram of flotation extraction apparatus: A, valve A; C, oil inlet; D, oil outlet; E, outlet; F, 3-way stopcock. Dimensions of paddle (inset) are in cm.

Extraction

Add ca 200 mL crushed ice to flotation flask and raise level of aqueous phase by adding 12.5% IMS from wash bottle until surface of aqueous phase is 1 cm below rim of flask. Lower paddle to ca 1 cm above valve A. Start geared motor (ca 100 rpm) and stir 3 min, or until ice has melted completely. Stop stirrer and apply suction to Hartley funnel. Run in kerosene slowly from reservoir D (filled to 600 mL) via inlet C until oil begins to overflow into gutter of flask and into Hartley funnel positioned below gutter outlet. Restart stirrer at its minimum speed (40–50 rpm) and increase revolutions gradually until all sample particles are just in motion. When 300 mL kerosene has flowed into flask, stop both flow of kerosene and stirrer. Raise stirrer until horizontal edge of paddle is 2–3 mm below lip of flask. Wash shaft of stirrer with 12.5% IMS until interface is just in contact with under-surface of paddle. Restart stirrer at its lowest revolution (40–50 rpm) and simultaneously add kerosene from outlet E, directing jet from 3 mm bore nozzle at junction between shaft and paddle blade. After remaining 300 mL kerosene has been used, dislodge any adhering mites from inside wall of flotation flask by means of fine jet of kerosene delivered from wash bottle. Stop paddle and add 12.5% IMS slowly to flotation flask from wash bottle until most of oil phase in flask passes over into Hartley funnel. Care should be taken to keep interface below lip of flask. Wash down gutter thoroughly with kerosene followed by 12.5% IMS. Stop suction.

Add 20 mL methylene blue solution (0.2 g in 1 L 12.5% IMS) to Hartley funnel; this stains filter paper deep blue. Thoroughly wash down sides of Hartley funnel and surface of filter paper with oil. Swirl Hartley funnel; this spreads mites across whole filter paper and facilitates examination and counting of mites. Restart suction and continue until filter paper is partially dried. Remove ring of Hartley funnel and carefully transfer filter paper to Petri dish. Examine filter paper and rim of ring under binocular microscope at magnification ranging between 20 and 50 ×. It is best to examine the filter paper immediately; however, it is possible to store filter paper (up to 1 week) for future examination by damping it with mixture of 12.5% IMS and glycerine (1 + 1).

Results and Discussion

The following procedure for preparing feed samples infested with known numbers of mites (*Acarus siro* L.) was used to test the performance of the improved method. Ten g portions of commercial concentrate dairy nuts, free from mites, were infested with 5, 10, 20, 50, 100, or 200 mites. The infested samples were sealed in a 5 × 2.5 cm glass tube and left on the bench for 24 h before extraction. Ten replicates for each level of infestation were examined.

The mean percentage recovery of mites from the 10 g samples of concentrate dairy nuts (Table 1) averaged between 84 and 96%. A regression analysis of data was done (Figure 3) using a method (2) for data where there is more than one value of Y for each value of X. To ensure that the data could be fitted to a straight line, the percentage values were transformed using the angular transformation. Calculating the variance ratio (F) of the mean square for the regression over the mean square for the deviation from the regression gives $F_{1,4} = 0.1699$, which is not significant at the 5% level. Therefore, the slope of the regression line does not differ significantly from zero and the number of mites present has no significant effect on the percentage recovery of mites within the range tested. The results obtained from this study differ from the

Table 1. Recovery of mites (*A. siro* L.) from 10 g sample of concentrate dairy nuts seeded with known number of mites

Number of mites added to each replicate	Recoveries										Mean recovery	Mean recovery, %
	1	2	3	4	5	6	7	8	9	10		
5	3	4	5	4	5	4	5	4	4	4	4.2	84
10	9	10	9	10	9	10	10	10	10	9	9.6	96
20	18	20	19	17	16	17	20	29	19	17	18.5	92.5
50	50	41	37	45	50	45	45	40	39	44	43.5	86.8
100	100	91	82	90	98	90	83	83	96	99	90.3	90.3
200	196	181	188	194	148	194	169	199	160	200	179.7	89.8

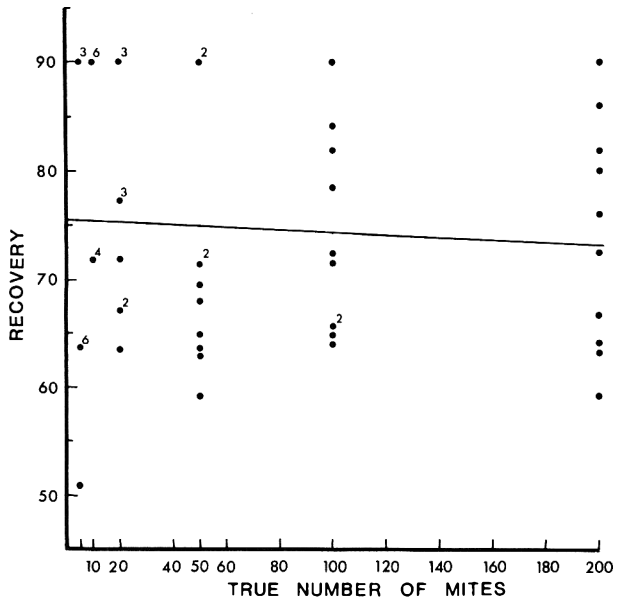

Figure 3. Regression line of percentage of mites (*A. siro* L.) recovered from 10 g dairy nuts at different levels of infestation. Percentage values were transformed using angular transformation.

conclusion reached by Thind and Griffiths (1), that the efficiency of the technique increases with the increase in infestation level. It is possible, however, that the slope of regression line obtained by them is distorted by the small number of replicates at the highest level of infestation. It may also be due to either the nature of the solvents employed or the types of material used as samples.

With the original method, certain types of animal feed samples including those used in this study consistently produced emulsions at the interface, which subsequently resulted in deposition of vegetative debris onto the ruled filter paper and made analysis difficult and time consuming. In this modified method, treating the sample with sodium chloride solution during the pre-extraction stage, followed by cooling the sample by adding crushed ice, has eliminated emulsion formation. Flushing mites from the interface into the oil layer, by introducing oil through a single 3 mm bore outlet E with simultaneous slow agitation of oil layer above the interface with the modified paddle, was more effective than was the perforated PVC ring used in the previous method. An important attribute of the modified method is that a substantially clean and readable filter paper is obtained; thus, only one filter paper per sample is required for accurate assessment of infestation level.

By incorporating staining of mites with Phloxin B in this method, the time taken for examination and counting of mites on the filter paper is further reduced as the mites stain cherry red and stand out against the blue background of the filter paper. The Phloxin B dye does not interfere with the specific identification of mites subsequently mounted on slides. In our experience, the time taken for counting varied between 4 and 10 min with mite infestation ranging between 500 and 20 000/kg. The use of alternative solvents in this method has not only helped in reducing the running costs but has also eliminated the unpleasant odor associated with the tetralin and kerosene mixture used in the original method. Although deodorized kerosene is now used as an oil phase, it is also possible to use domestic paraffin (s.g. = 0.775–0.795) without any detrimental effect on the efficiency of the technique.

The modified method provides a reliable and rapid technique for estimating mite populations in a wide variety of materials, including those listed in the original method (1). It is also recommended that a 10 g sample weight be used for compacted feedingstuffs. The improved methodology reduces the time taken to carry out 4 extractions simultaneously (using a battery of 4 flotation flasks) from 1 h to 35 min.

References

(1) Thind, B. B., & Griffiths, D. A. (1979) *J. Assoc. Off. Anal. Chem.* **62**, 278–282

(2) Sokal, R. R., & Rohlf, F. J. (1969) *Biometry*, W. H. Freeman and Co., San Francisco, CA, pp. 430–432

FEEDS

Comparison of HgO and $CuSO_4$ as Digestion Catalysts in Manual Kjeldahl Determination of Crude Protein in Animal Feeds: Collaborative Study

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The official AOAC manual Kjeldahl method for determining crude protein in animal feeds, 7.015, uses HgO as a catalyst in the digestion step. Because of environmental considerations, there is considerable interest in alternative catalysts. A collaborative study compares the official HgO-catalyzed method and an alternative using $CuSO_4$. Fifty-four samples consisting of blind duplicates of closely matched pairs, representing a range of animal feed materials and 2 standard materials, were analyzed once by each method. Results were returned by 22 laboratories. Means and standard deviations between methods were comparable. The $CuSO_4$-catalyzed method has been adopted official first action.

The official manual Kjeldahl method for determining crude protein in animal feeds, **7.015** (1), uses HgO as a catalyst in the digestion procedure. In practice, however, various laboratories have substituted other catalysts or mixtures of catalysts; $CuSO_4$ is probably the most popular alternative. Not only do laboratories vary in the catalyst used, but apparently also vary significantly in the digestion parameters of time, heat input, and salt–acid ratio.

The issue of copper vs mercury as a catalyst was addressed by the Associate Referee in 1954 (2) and by a joint AOAC–American Oil Chemists' Society Committee in 1955 (3). In both studies, the comparison was made by using the established mercury catalyst procedure and keeping all digestion parameters constant while changing only the 2 catalysts.

The 1954 study reported equivalent results by both catalysts on all samples except nicotinamide, for which the copper catalyst produced significantly lower results. The recommendation was to allow either catalyst except when refractory nitrogen compounds were suspected to be present.

The 1955 study reported small but significant differences between the means of copper and mercury results, and larger standard deviations for the copper version of the method. The recommendation was to discontinue the use of $CuSO_4$ as an alternative catalyst in the official methodology.

The issue is still unresolved however, because a significant number of laboratories continue to express confidence in the copper catalyst version. The issue has become particularly acute in recent years because of the environmental concerns associated with mercury.

In 1976, Rexroad and Cathey (4) reported on efforts to optimize the digestion parameters specifically for the $CuSO_4$ catalyst. That method is the basis for this collaborative study.

Fifty-four samples were used in the collaborative study. They consisted of 26 blind duplicates arranged as 13 closely matched pairs, plus 2 standard materials. The sample materials were to be analyzed once each by the official method, **7.015** (1), and by the 1976 $CuSO_4$ version as presented below. Collaborators were also asked to perform boil tests on their digestion heaters and report the results of those tests.

Protein (Crude) in Animal Feed
Copper Catalyst Kjeldahl Method
First Action

(*Caution: See* **51.030, 51.037.**)

Principle

Sample is digested in H_2SO_4, using $CuSO_4$ as catalyst, converting N to NH_3 which is distd and titrd.

Reagents

(a) *Sodium hydroxide.*—Pellets, flakes, or soln with sp. gr ≥1.36, low N. Dissolve ca 450 g NaOH in H_2O cool, dil. to 1 L.

(b) *Alundum.*—Boiling stones, 8–14 mesh (Arthur H. Thomas Co., No. 1590-D18).

Table 1. Collaborative samples for Kjeldahl digestion comparison

Closely matched pair No.	Sample No. of blind duplicate	Approx. % protein	Material
1	1,28	18	dehydrated alfalfa meal
	11,29	17	dehydrated alfalfa meal
2	2,23	88	soy protein concentrate
	7,37	89	soy protein concentrate
3	3,39	17	pullet grower
	15,27	15	layer
4	4,26	55	meat and bone meal
	17,43	54	meat and bone meal
5	5,54	11	custom mix cattle feed
	25,51	10	beef feed
6	6,44	30	swine base mix
	10,33	29	swine base mix
7	8,46	27	dry dog food
	14,36	29	dry puppy food
8	9,38	86	hydrolyzed poultry feathers
	13,42	89	hydrolyzed poultry feathers
9	16,45	41	cottonseed meal
	19,41	41	cottonseed meal
10	18,30	84	blood meal
	34,40	82	blood meal
11	12,35	16	swine developer
	21,49	17.5	swine grower
12	22,50	18	milk replacer
	31,52	17	milk replacer
13	24,53	44	soybean meal
	32,48	44	soybean meal
	20	78	NBS SRM $NH_4H_2PO_4$
	47	92	Eastman lysine.HCl, Lot A5X

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This report of the Associate Referee was presented at the 97th Annual International Meeting of the AOAC, Oct. 3–6, 1983, at Washington, DC.
The recommendations of the Associate Referee were approved by the General Referee and Committee A and were adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) 67, March issue.

Table 2. Collaborative Results

Collaborator	#1		#2		#3		#4	
Sample #	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result
1	18.00	18.90	18.77	18.85	17.56	18.12	18.85	19.23
2	88.37	88.18	87.56	87.35	87.50	88.75	88.74	89.18
3	15.74	15.68	15.72	15.65	15.75	14.50	15.91	15.98
4	54.16	54.22	54.20	53.84	51.25	51.50	54.95	54.30
5	13.68	13.23	13.32	13.54	13.18	13.06	13.55	13.55
6	29.86	30.13	29.76	29.65	30.00	25.87	29.67	30.52
7	89.76	90.36	88.21	86.95	89.50	87.25	90.05	90.63
8	22.63*	27.17	26.86	26.90	26.50	26.87	26.89	27.37
9	86.55	87.16	85.59	84.78	88.00	88.25	87.01	87.37
10	29.21	29.55	29.42	29.28	29.06	27.75	29.74	28.44
11	17.01	17.06	16.93	16.75	16.75	16.56	17.02	17.02
12	16.92	17.16	16.79	16.68	16.81	16.81	17.08	17.22
13	90.78	89.86	89.92	89.41	88.75	81.50	91.68	91.64
14	28.31	28.09	28.28	28.00	28.00	26.37	28.40	28.62
15	16.62	16.44	16.37	16.25	16.25	15.37	16.48	16.19
16	39.02	39.50	39.26	39.05	39.37	38.87	40.05	39.95
17	54.14	53.80	53.69	53.19	52.50	51.50	54.55	54.56
18	85.83	85.73	84.25	83.88	84.75	88.00	84.79	84.73
19	40.35	40.39	41.18	39.90	40.37	41.12	40.27	41.08
20	76.00	75.48	75.18	75.29	80.50	72.25	77.10*	74.72
21	17.74	18.06	17.92	17.60	17.87	20.12	18.03	18.18
22	22.08*	17.62	17.74	17.48	18.00	15.75	17.93	17.95
23	88.52	88.32	86.29	87.46	89.50	85.50	88.68	88.86
24	44.41	44.21	44.27	45.00	41.37	41.87	44.45	44.67
25	10.22	10.02	10.12	10.11	10.05	9.93	10.25	10.40
26	54.81	54.19	53.41	53.79	51.75	51.00	54.94	50.24*
27	16.46	16.06	16.06	16.75	16.12	15.18	16.46	16.33
28	18.63	18.70	18.73	19.04	19.37	18.37	18.85	18.92
29	17.17	17.05	16.74	16.73	15.93	15.31	16.91	17.01
30	85.97	85.51	83.83	85.57	81.50	81.00	85.94	85.88
31	19.91*	17.71	17.60	17.89	17.25	16.12	18.04	18.15
32	44.89	45.37	43.22	44.61	45.12	44.25	44.90	45.22
33	29.20	29.77	29.42	29.48	29.37	28.00	29.32	29.51
34	81.01	81.41	81.00	80.12	80.25	83.00	81.37	81.41
35	17.32	17.07	17.25	17.06	15.56	15.50	17.16	17.21
36	28.09	28.31	28.06	28.14	28.12	27.18	28.29	28.54
37	89.99	89.80	89.84	89.31	88.00	86.50	89.55	90.27
38	86.43	86.31	86.03	85.10	86.56	81.50	85.96	85.55
39	15.42	15.66	15.69	15.77	15.62	14.87	15.89	15.52
40	81.15	81.41	80.72	79.46	78.25	75.75	80.83	81.81
41	40.48	40.20	41.04	39.93	39.25	37.62	40.28	40.54
42	90.70	90.03	90.59	90.27	86.50	89.00	91.44	91.62
43	53.84	54.24	53.84	53.98	55.00	52.75	54.05	54.60
44	29.98	24.44*	29.95	29.89	29.43	30.00	29.87	29.98
45	39.95	40.21	39.26	39.25	39.87	40.00	39.50	40.25
46	27.36	27.06	26.88	27.15	26.93	25.06	27.18	27.26
47		92.54	95.15	95.36	86.50	93.50	85.38*	96.85
48	45.02	44.89	45.13	43.98	44.37	41.62	44.91	44.90
49	17.93	18.19	17.89	17.78	17.43	16.93	18.23	18.13
50	17.88	17.52	17.49	17.34	17.81	16.81	17.89	18.10
51	9.70	10.20	9.92	10.12	10.06	9.62	10.34	10.17
52	17.65	17.88	17.82	17.71	17.56	17.18	17.81	18.09
53	43.41	44.41	43.85	43.26	44.75	42.37	44.00	44.27
54	13.42	13.36	13.49	13.59	13.25	13.25	13.45	13.90

(c) *Methyl red indicator.*—Dissolve 1 g Me red (Na salt) in 100 mL MeOH.

(d) *Hydrochloric or sulfuric acid std soln.*—0.5N. Prep. as in **50.011–50.017** or **50.039–50.040.**

(e) *Sodium hydroxide std soln.*—0.1N. Prep. as in **50.032–50.035.**

After stdzg both acid and base by methods suggested in (d) and (e), also check cne against the other. In addn, check entire method by analyzing NBS Std Ref. Material No. 194, $NH_4H_2PO_4$, certified 12.15% N, and high purity lysine.HCl.

Apparatus

(a) *Digestion.*—Use Kjeldahl flasks with capacity of 500–800 mL.

(b) *Distillation.*—Use digestion flask (Corning No. 2020, for example) connected to distn trap by rubber stopper. Distn trap is connected to condenser with low-S tubing. Outlet of condenser tube should be <4 mm diam.

Determination

Weigh 0.250–1.000 g sample into digestion flask. Add 15 g K_2SO_4, 0.04 g anhyd. $CuSO_4$, 0.5–1.0 g alundum granules,

Table 2 cont. Collaborative Results

Collaborator	#5		#6		#7		#8	
Sample #	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result
1	18.71	18.91	19.04	18.92	19.25	19.12	18.72	18.41
2	87.24	86.04	88.11	88.53	88.42	88.46	87.00	86.98
3	15.10	15.90	15.95	16.25	15.72	15.85	15.28	15.65
4	54.16	53.36	54.88	54.64	54.94	54.58	54.30	53.85
5	13.30	13.70	13.60	13.64	13.52	13.47	13.16	13.27
6	29.21	29.01	30.49	30.40	29.85	29.93	29.31	30.05
7	89.04	87.64	89.47	89.93	89.88	89.66	88.89	89.43
8	26.01	26.21	27.39	27.40	27.33	26.95	27.03	26.94
9	85.44	85.24	86.83	86.28	86.40	86.22	85.49	85.46
10	28.71	29.21	29.58	29.54	29.59	29.43	29.36	29.26
11	16.40	16.90	16.79	17.09	17.10	16.97	17.24	16.09
12	17.00	17.30	17.34	17.26	17.09	17.08	17.00	17.10
13	87.24	87.84	90.39	91.09	90.79	90.46	88.58	88.18
14	27.71	27.61	28.53	28.54	28.49	28.19	27.84	28.22
15	16.00	16.40	16.36	16.34	16.21	16.09	15.17	16.18
16	39.22	39.22	39.84	39.98	40.01	40.07	35.91*	36.17*
17	54.16	53.60	54.19	54.13	54.16	53.66	53.36	53.20
18	83.04	82.64	85.16	85.73	85.39	85.48	85.01	84.88
19	38.32	39.82	41.42	40.70	39.82	40.13	39.14	39.16
20	74.83	74.63	75.57	76.06	75.76	75.74	75.39	74.78
21	17.71	17.50	18.16	18.57	17.83	17.86	17.53	17.70
22	17.71	17.50	18.08	17.83	17.84	17.81	17.73	17.73
23	87.04	87.24	89.34	88.40	88.33	88.15	87.48	86.65
24	43.02	43.82	44.41	44.17	44.25	44.65	43.72	44.48
25	10.10	9.90	10.31	10.30	10.34	10.14	10.11	9.91
26	52.82	53.71	54.69	54.47	54.66	54.72	54.06	53.61
27	15.90	15.60	16.36	16.54	16.34	16.34	15.49	15.92
28	18.71	18.91	19.02	19.02	18.89	18.85	18.62	18.62
29	16.70	16.70	16.91	16.97	16.85	16.84	17.93*	17.27
30	83.44	82.44	85.71	85.75	85.79	85.63	85.14	85.26
31	17.50	17.10	18.05	18.09	18.06	18.40	17.84	17.82
32	44.32	43.92	44.42	44.56	44.91	44.38	45.69	44.17
33	28.91	28.81	29.68	29.62	29.57	29.28	29.34	29.44
34	80.24	80.04	81.37	81.21	81.09	81.00	80.65	80.75
35	16.80	16.90	17.38	17.15	17.11	17.21	16.87	17.03
36	27.31	27.61	28.28	28.40	28.33	28.22	28.27	27.15
37	88.84	88.84	89.30	89.44	89.71	89.88	84.64*	87.64
38	83.64	85.44	86.39	86.77	86.58	86.47	85.31	83.15
39	15.70	15.70	15.96	16.02	15.72	15.72	15.68	15.64
40	80.44	80.44	80.91	81.23	80.93	81.16	80.05	80.59
41	39.22	39.52	40.24	39.96	41.31	40.59	40.53	39.93
42	89.84	87.84	90.93	90.89	90.65	90.82	87.73	89.39
43	53.04	51.71	55.44	54.45	54.52	54.09	50.97*	53.64
44	29.31	29.41	30.01	30.07	30.00	29.62	29.26	29.97
45	38.55	39.42	39.51	39.86	39.69	40.46	39.96	37.75
46	26.81	26.71	27.42	27.44	27.16	26.96	27.14	26.49
47	92.04	92.04	95.58	95.00	95.17	95.38	93.56	93.01
48	43.62	44.42	44.76	44.84	44.59	44.09	42.46	44.95
49	17.91	18.01	17.95	18.26	18.07	18.10	18.24	17.89
50	17.81	17.31	17.83	17.99	17.97	17.87	16.42*	17.43
51	10.00	10.00	10.40	10.27	10.11	10.25	10.14	9.94
52	17.61	17.70	17.86	18.07	18.09	17.97	17.75	17.76
53	43.92	43.92	44.62	44.10	44.07	44.06	44.52	43.55
54	13.20	13.50	13.44	13.74	13.45	13.60	13.79	13.84

and 20 mL H_2SO_4. (Add addl 1.0 mL H_2SO_4 for each 0.1 g fat or 0.2 g other org. matter if sample wt is >1 g.)

Include at least one sample of high purity lysine.HCl in each day's run as check of correctness of digestion parameters. If recovery is not complete, make appropriate adjustments.

Heat flask at 5 min boil rate (burner preheated and adjusted to bring 250 mL H_2O at 25° to rolling boil in 5 min) until dense white fumes clear bulb of flask, swirl gently, continue heating addnl 90 min. (Note: Reagent proportions, heat input, and digestion time are critical factors—do not change.) Cool, cautiously add 250 mL H_2O, and cool to room temp. (Note: If bumping occurs during distn, vol. of H_2O may be increased to ca 275 mL.)

Prep. titrn beaker by adding accurately measured appropriate vol. std acid soln to amt of H_2O such that condenser tip will be sufficiently immersed. Add 3–4 drops indicator soln (c).

Add 2–3 drops of tributyl citrate in digestion flask to reduce foaming; add another 0.5–1.0 g alundum granules. Slowly down side of flask, add sufficient NaOH soln (a) such that mixt. will be strongly alk. Immediately connect flask to distn

Table 2 cont. Collaborative Results

Collaborator	#9		#10		#11		#12	
Sample #	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result
1	18.81	19.13	19.05	18.81	18.26	18.60	18.38	17.81
2	87.62	87.62	87.98	87.70	86.26	87.19	88.88	88.56
3	15.81	15.75	15.82	15.57	15.36	15.50	15.50	15.63
4	54.38	54.06	54.49	54.03	53.88	53.88	54.25	53.75
5	13.19	13.44	13.62	13.72	13.14	13.13	12.75	13.75
6	29.75	29.69	29.34	29.26	29.63	29.68	29.00	28.50
7	87.38	88.76	90.16	89.75	88.41	88.86	86.75	86.94
8	26.69	27.19	27.29	27.19	26.72	27.05	27.13	26.50
9	85.26	85.62	86.62	86.10	85.19	85.88	81.50*	82.69
10	29.06	28.56	29.51	29.62	29.15	29.33	28.13	28.25
11	17.13	16.88	17.13	16.98	16.79	16.81	16.44	16.75
12	16.75	16.75	17.40	17.27	16.92	17.11	16.88	16.88
13	90.76	90.62	91.14	90.61	89.98	90.17	92.25	91.50
14	27.81	28.06	28.15	28.39	28.07	27.97	27.75	27.06
15	16.38	16.25	16.35	16.49	16.92	16.68	15.88	16.00
16	39.13	39.44	39.77	39.76	39.52	39.57	38.00	38.06
17	53.81	53.75	54.65	54.00	54.05	53.71	54.13	53.25
18	85.50	85.50	85.42	86.03	85.14	85.18	83.75	82.94
19	40.75	40.88	41.65	41.07	41.43	40.53	39.50	39.18
20	75.76	75.76	75.87	75.80	74.91	75.38	76.13	75.88
21	17.81	18.13	18.16	18.08	18.09	17.82	17.81	18.31
22	17.56	17.88	17.95	17.86	17.74	17.86	17.38	17.00
23	88.62	89.50	88.29	87.90	85.89	87.46	87.75	86.44
24	43.88	44.50	44.28	44.24	43.51	44.25	44.31	43.88
25	10.06	10.38	10.41	10.31	9.89	10.16	9.75	9.63
26	53.75	54.44	54.13	54.38	53.88	54.50	53.88	54.00
27	15.75	16.63	16.42	16.37	15.68	16.06	16.38	15.38
28	18.50	18.81	18.86	18.90	18.12	18.03	17.44*	17.56
29	16.88	17.25	16.77	17.18	16.77	17.16	16.19	16.38
30	83.50	85.62	85.74	85.60	84.49	85.22	84.50	84.00
31	17.56	17.94	17.97	18.03	17.42	17.99	18.00	18.19
32	44.69	44.50	44.00	45.05	44.51	45.35	44.38	43.81
33	29.38	29.38	29.41	29.35	29.01	29.02	29.25	29.63
34	81.26	81.26	82.57	81.16	79.27	80.02	76.63*	76.19*
35	16.81	17.13	17.07	17.35	16.94	17.14	16.63	16.63
36	27.69	28.00	28.24	28.39	27.92	28.32	28.44	27.50
37	88.62	89.50	89.61	89.57	88.33	89.30	89.38	86.88
38	86.12	85.88	86.88	86.38	84.49	86.00	87.50	87.13
39	15.63	15.94	15.86	15.97	15.46	15.79	16.00	16.38
40	79.76	80.38	80.82	81.08	79.97	80.19	79.50	79.63
41	39.56	40.31	39.99	40.71	40.64	40.65	40.63	40.94
42	89.62	90.12	90.56	90.78	89.63	90.39	90.13	89.25
43	53.94	54.19	54.39	54.21	53.75	54.14	52.13	51.38
44	29.31	30.00	30.30	30.37	29.88	30.19	28.56	29.06
45	39.75	39.94	39.47	39.90	39.33	39.95	38.69	38.44
46	26.50	26.56	27.38	27.27	26.79	26.81	26.25	26.25
47	91.50	94.50	94.44	95.74	93.99	94.68	93.75	93.94
48	43.94	44.75	45.02	44.62	44.73	44.86	44.56	44.19
49	17.75	18.13	18.06	17.96	17.77	17.74	17.94	17.75
50	17.69	17.88	17.98	17.81	17.55	17.66	17.75	17.38
51	10.00	10.19	10.48	10.42	10.24	9.98	10.94*	9.75
52	17.69	17.88	17.60	18.10	17.38	17.92	16.88*	16.38*
53	44.25	44.13	44.62	43.82	43.82	44.21	44.31	43.94
54	13.44	13.56	13.58	13.68	13.50	13.49	12.63	13.63

app., mix completely, and distil at ca 7.5 min boil rate until ≥150 mL distillate is collected in titrn beaker.

Titr. excess std acid in distillate with std NaOH soln. Correct for blank detn on reagents. Calc. % N:

$$\% N = [(N_{acid})(mL_{acid}) - (mL_{bk})(N_{NaOH}) - (mL_{NaOH})(N_{NaOH})][1400.67]/mg\ sample$$

where mL_{NaOH} = mL std base needed to titr. sample; mL_{acid} = mL std acid used for that sample; mL_{bk} = mL std base needed to titr. 1 mL std acid minus mL std base needed to titr. reagent blank carried thru method and distd into 1 mL std acid; N_{acid} = normality of std acid; N_{base} = normality of std base. Calc. % crude protein, defined as 6.25 × % nitrogen, or 5.7 × % nitrogen for wheat grains.

Results and Discussion

Table 1 identifies the sample varieties sent to collaborators. In general, closely matched pairs were obtained from the same manufacturing source, if possible, different lots of the same product. Having blind duplicates eliminates the possibility of biased results because the collaborators cannot dis-

Table 2 cont. Collaborative Results

Collaborator	#13		#14		#15		#16	
Sample #	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result
1	18.02	17.86	18.85	18.70	18.88	18.97	19.24	18.79
2	85.58	85.01	87.60	87.00	88.22	88.05	91.28	89.31
3	14.90*	14.57*	15.60	15.45	15.57	15.60	16.37	16.46
4	53.05	52.05*	53.75	50.45*	54.37	54.19	55.48	57.27
5	12.55	12.60	13.15	13.05	13.31	13.18	14.32	13.87
6	29.20	28.34	29.70	29.30	29.76	29.81	31.17	30.30
7	86.57	86.42	88.45	88.70	89.42	89.63	91.28	91.28
8	25.99	26.19	26.85	26.45	27.16	26.94	27.74	27.29
9	83.37	83.36	85.70	85.20	86.04	86.19	89.31	88.96
10	28.37	28.89	29.00	28.75	29.83	29.21	29.76	29.31
11	16.28	16.18	16.80	16.85	16.79	16.88	17.16	17.51
12	16.60	16.54	16.85	16.95	17.03	16.92	17.51	17.78
13	87.01	87.78	90.10	89.70	90.13	89.77	93.96	93.07
14	27.00	27.14	27.90	26.80	28.13	28.15	29.53	28.90
15	15.36	15.44	16.10	16.00	16.04	16.03	16.56	16.33
16	38.61	38.66	39.50	38.70	39.40	39.43	41.17	42.06
17	52.59	52.37	53.30	50.45	54.21	53.75	55.04	57.27
18	82.31	81.55	84.80	84.40	85.42	85.22	88.96	89.49
19	38.09	39.00	40.70	40.50	40.66	40.47	42.06	42.06
20	73.02*	73.50*	74.90	74.60	75.91	75.80	77.06	79.65
21	17.05*	17.20	17.95	17.80	17.43	17.73	18.79	18.35
22	16.86	16.80	17.65	17.30	17.72	17.79	17.78	18.12
23	85.67	85.41	87.40	87.50	88.07	87.65	90.38	91.28
24	42.74	43.18	42.90	44.35	44.10	43.77	45.64	46.09
25	9.71	9.57	10.15	10.00	10.03	9.96	10.77	10.68
26	52.77	52.41	53.90	51.55	54.47	54.17	55.48	55.48
27	15.25	15.58	16.05	16.00	16.10	16.11	16.78	16.56
28	18.25	17.98	18.90	18.85	18.90	18.80	19.24	19.24
29	16.41	16.19	16.85	16.95	16.90	16.95	17.16	17.60
30	82.96	82.38	84.70	84.90	85.45	85.14	89.49	89.31
31	17.48	17.21	17.90	17.80	17.80	17.82	18.35	18.35
32	43.51	43.38	43.60	44.20	45.02	45.02	45.53	46.53
33	28.25	27.92	29.30	29.20	29.50	29.24	31.77	30.65
34	77.63*	77.23*	80.30	79.40	80.43	80.57	84.12	85.01
35	16.45	16.37	16.90	16.80	16.79	16.84	17.25	17.69
36	27.32	27.18	28.05	27.50	28.25	28.02	28.64	28.64
37	86.46	82.36*	89.00	88.50	89.79	89.35	90.38	91.28
38	84.26	83.40	85.30	85.50	86.08	85.97	90.72	93.07
39	15.21	15.07	15.65	15.35	15.68	15.65	16.33	16.02
40	78.32	77.57	80.20	80.20	80.52	80.32	84.12	83.22
41	38.56	38.75	40.85	39.90	39.97	40.17	44.74	42.51
42	87.81	87.62	90.10	89.60	90.49	90.21	93.07	93.07
43	52.49	52.30	53.10	49.05*	53.96	53.90	55.93	55.04
44	28.97	29.11	30.00	29.05	30.17	29.88	30.87	29.98
45	38.65	38.34	39.25	38.55	39.49	39.26	41.61	40.63
46	26.17	25.97	26.95	26.65	27.07	26.88	27.85	27.49
47	92.35	91.59	89.70*	94.50	95.47	95.40	95.75	96.65
48	42.92	42.42*	44.10	44.55	44.54	44.42	45.64	46.53
49	17.47	17.32	17.95	14.95	17.89	17.83	18.79	18.57
50	17.29	17.13	17.65	17.50	17.66	17.72	18.45	18.35
51	9.70	9.76	9.90	9.90	10.05	10.02	10.77	10.86
52	17.34	17.16	17.75	17.50	17.68	17.59	18.35	17.90
53	42.92	42.56*	44.10	43.75	44.16	43.98	45.64	45.64
54	12.96	12.51*	13.40	13.45	13.64	13.30	14.09	14.09

tinguish blind duplicates from closely matched pairs. Collaborative results were received from 22 laboratories (Table 2).

There were a number of comments from collaborators. Three mentioned that they experienced some degree of bumping during distillation, and suggested adding more than the specified 250 mL water after digestion. Bumping may be a function of flask size because not all analysts experienced the problem. One person commented on the fine quality of the sample grind, while another thought some samples could have been more finely ground.

Collaborator 6 noted some problems with caking of the digest when 5 min boil heat input was used. Collaborator 13 substituted BDH Chemicals Ltd aluminum oxide (4–8 mesh) for alundum, and felt it was better to weigh the feeds into tissue paper, roll the paper up, and drop it into the flasks. Collaborator 18 changed the standard acid concentration to 0.1N and reduced the minimum sample size to 0.14 g for the samples of highest protein content. Collaborator 15 added 0.2 mL saturated aqueous solution of $CuSO_4$.

Collaborator 14 made several observations. He suggested that the method write-up should specify the digestion times (2 h for mercury and 90 min for copper). He suggested that the positive bias for copper might be attributed to co-precipitation of ammonia with mercury in the mercury method. He

Table 2 cont. Collaborative Results

Collaborator	#17		#18		#19		#20	
Sample #	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result
1	18.91	19.02	19.13	19.26	18.63	18.56	17.96	18.40
2	87.53	87.69	87.50	86.28	85.73	86.03	86.55	87.07
3	16.00	16.00	15.79	15.83	15.67	15.56	15.33	14.89
4	54.46	54.49	54.75	54.50	53.91	53.15	52.74	53.87
5	13.66	13.72	13.39	13.28	13.47	13.37	13.05	12.88
6	29.77	29.99	30.95	29.70	29.39	29.43	28.73	29.08
7	89.32	89.52	88.37	87.32	86.48	86.77	84.80	87.60
8	27.28	27.07	27.00	26.57	26.68	26.89	26.72	27.16
9	68.38*	68.57*	85.24	84.62	86.37	85.27	85.67	86.11
10	29.45	29.71	29.35	29.57	28.83	29.17	28.73	28.65
11	17.28	17.49	17.05	16.78	16.65	16.40	16.12	16.56
12	17.29	17.42	16.97	17.19	16.82	16.74	16.38	16.64
13	90.70	90.70	90.35	89.89	89.20	88.24	88.30	89.35
14	28.54	28.49	28.27	28.12	27.81	27.54	27.16	28.03
15	15.97	16.07	16.27	16.43	15.96	15.74	14.89	16.16
16	38.99	38.74	38.98	39.52	39.21	38.90	37.49	38.76
17	54.04	54.23	54.09	53.80	53.70	52.92	55.71	53.39
18	85.20	85.60	85.70	85.23	85.18	84.78	81.47	85.32
19	41.09	41.27	40.36	41.63	41.04	40.91	40.21	40.69
20	75.96	75.75	75.58	75.35	78.16*	78.14*	72.88	76.21
21	18.18	18.07	18.31	18.13	17.70	17.43	19.27	19.14
22	18.04	17.86	18.03	17.81	17.50	17.66	18.13	18.35
23	88.16	88.43	87.23	88.29	86.36	87.26	91.98	91.81
24	44.41	44.44	43.60	44.17	44.64	43.80	48.18	48.27
25	10.40	10.33	10.16	9.81	10.20	10.00	11.39	11.21
26	54.45	54.45	54.22	54.37	54.40	54.05	54.84	55.36
27	16.46	16.59	15.61	16.34	16.51	15.96	17.26	17.52
28	19.00	19.26	19.09	19.23	18.62	18.67	19.10	19.18
29	16.98	17.19	16.99	17.24	16.57	16.77	17.35	17.74
30	84.92	85.17	85.12	85.64	84.59	83.99	87.60	89.70
31	18.04	18.21	17.76	17.95	18.07	17.94	17.96	18.13
32	45.05	45.18	45.16	45.11	43.60	43.32	49.06	49.71
33	29.56	29.75	29.51	29.28	29.26	29.28	29.43	29.96
34	81.42	81.63	80.61	80.49	80.00	79.59	90.05	84.36
35	17.26	17.47	17.23	17.10	17.02	17.00	18.92	18.97
36	28.54	28.52	28.49	27.82	27.79	27.91	29.35	29.04
37	89.65	89.44	88.33	89.70	88.16	87.62	89.18	90.67
38	86.43	86.66	83.80	86.42	85.00	86.17	90.05	89.18
39	15.51	15.58	15.94	15.47	15.84	15.62	17.34	16.95
40	81.37	81.70	81.00	81.58	80.08	80.00	89.88	88.39
41	42.90	42.74*	41.34	43.19*	40.25	40.00	42.05	43.14
42	90.56	91.22	90.78	90.47	90.29	90.52	92.51	92.42
43	54.43	54.59	53.95	53.54	53.00	53.73	55.36	55.41
44	30.34	30.64	30.58	29.96	29.28	28.94	29.70	30.79
45	39.45	39.33	41.13	39.54	38.45	39.11	40.65	40.69
46	27.42	27.49	27.76	26.89	26.63	26.75	28.03	28.91
47	94.77	95.78	95.12	95.66	92.09	92.58	90.93	94.08
48	45.28	45.49	43.11	44.84	43.53	44.28	42.49	44.24
49	18.32	18.56	18.07	20.27	17.53	17.86	17.43	17.52
50	18.14	18.37	17.91	17.82	17.38	17.65	17.26	17.65
51	10.51	10.26	12.48*	10.15	9.95	9.99	9.46	9.64
52	18.03	18.00	18.06	18.00	17.41	17.54	16.99	17.52
53	44.58	44.81	44.19	44.27	43.33	43.34	42.92	43.80
54	13.31	13.41	13.64	13.39	13.35	13.26	12.70	13.27

noted that while lysine.HCl has a sharp titration end point in the mercury method, he regularly observed an indefinite end point with that one material in the copper method.

Two-sample X-Y charts were prepared according to Youden (5). Blind duplicates were plotted as additional data on the same chart. The charts were examined for gross outliers. Eleven of the 22 laboratories had at least one obvious outlier (Table 3).

Three laboratories, 3, 16, and 20, reported gross outlying values for about half of their 52 possible pairs of results. Laboratories 16 and 20 had consistently high results, while Laboratory 3 had consistently low results. In conversation with those analysts, no obvious causes of the bias could be pinpointed. Nevertheless, those 3 laboratories were omitted from further statistical consideration.

The remaining data were processed for outliers according to Steiner (6). Blind duplicates were considered as the same sample, making a total of 38 data points for each sample. In a few cases, when there was a rather gross outlier which excessively distorted the standard deviation, a second pass through the data was made after omitting that outlier to see if additional data could be declared outliers.

Two samples, 20 and 47, were standard materials which did not have blind duplicates. These consisted of only 19 data

Table 2 cont. Collaborative Results

Collaborator	#21		#22	
Sample #	$CuSO_4$ Result	HgO Result	$CuSO_4$ Result	HgO Result
1	18.9	18.8	19.16	18.93
2	88.7	88.5	88.85	86.32
3	15.9	15.7	15.96	15.38
4	55.0	54.7	54.78	53.85
5	13.4	13.6	13.42	15.32*
6	30.1	30.0	30.22	29.76
7	90.3	90.2	88.08	86.67
8	27.4	27.2	26.92	26.36
9	86.9	86.6	85.34	79.17*
10	29.6	29.5	29.23	28.25
11	17.2	17.0	17.14	15.97
12	17.1	17.4	16.66	16.18
13	91.3	91.2	90.46	90.09
14	28.4	28.4	28.47	27.98
15	16.2	16.4	16.80	16.01
16	39.9	39.8	40.34	40.31
17	54.2	54.2	53.76	54.91
18	86.1	86.2	83.78	87.64
19	40.8	40.4	39.85	41.09
20	75.9	75.9	76.18	75.89
21	18.2	18.1	18.37	18.06
22	18.0	18.0	17.94	17.79
23	88.5	88.3	87.41	88.87
24	44.3	44.6	43.84	43.75
25	10.3	10.2	10.28	9.29*
26	54.5	54.6	53.37	53.91
27	16.5	16.2	15.88	16.33
28	19.1	19.1	18.76	18.43
29	17.1	17.0	16.57	16.92
30	85.8	85.8	83.67	84.91
31	18.2	18.0	17.92	17.63
32	45.1	44.7	44.67	44.65
33	29.7	29.6	29.63	30.09
34	81.2	81.5	82.16	81.47
35	17.3	17.2	17.19	16.92
36	28.4	28.4	28.27	28.38
37	90.4	90.1	88.99	89.57
38	86.7	86.6	86.07	86.47
39	15.9	15.8	15.79	15.89
40	81.4	81.4	81.56	81.40
41	41.0	41.1	39.93	40.11
42	91.3	91.2	90.77	91.35
43	54.1	54.1	53.45	54.03
44	30.1	30.2	29.98	29.98
45	40.8	40.8	38.88	39.70
46	27.3	27.2	26.84	27.28
47	94.6	95.8	94.76	95.82
48	45.2	45.2	44.45	45.37
49	18.1	18.2	17.67	17.82
50	17.9	17.9	17.82	17.67
51	10.2	10.2	9.94	10.09
52	17.7	18.0	17.88	18.05
53	44.6	44.5	44.30	44.79
54	13.6	13.6	13.10	13.19

points each, so outliers were checked using the Dixon test (7).

Outliers are noted by an asterisk in Table 2. There were 23 copper catalyst outliers and 22 mercury catalyst outliers, representing 2.2% of the total data for 19 laboratories.

The remaining data, with outliers removed, were processed for information on precision by using Youden's scheme for closely matched pairs (8). Because the collaborative scheme included blind duplicates of the closely matched pairs, there were 2 blind duplicates for each X and Y pair from each laboratory. Table 4 contains the various X and Y means and

Table 3. Gross outliers from X-Y charts

Lab.	No. of visual outliers
-	3
3	25
4	1
8	8
12	9
13	13
14	4
16	26
18	2
20	24
22	2

the standard deviations: S_r, the estimate of random error, S_b, the estimate of interlaboratory bias, and S_d, the estimate of overall precision.

Using this table, it is possible to compare the precision between the mercury and the copper versions of the method. In general, the various standard deviations for the 2 methods compare reasonably closely by sample set. Of the 39 possible pairs of standard deviations, mercury had the largest 24 times and copper 15 times. However, in only one sample set, 12,35–21,49, did the mercury method standard deviations appear noticeably large compared with those of the copper method.

The grand means of blind duplicates were slightly higher for the copper method in 17 sample sets, and for the mercury method in 9 sample sets. The largest difference was about 0.2%, but most means were in much closer agreement. The overall average difference, copper minus mercury grand means, was +0.015%.

It should be noted that the comparisons of both precision and mean were just the opposites of what was found in the 1955 study (3). This can be attributed to the optimization of digestion parameters for copper in the current version of the method.

To get a rough idea of the expected precision of the methods at a given protein level, the data from Table 4 were grouped by level in 10% increments (Table 5). The standard deviations within each 10% increment were combined by averaging the variances. Of course, the nature of the sample or its preparation before analysis might also be significant factors in a particular case.

Sample 20, NBS SRM $NH_4H_2PO_4$, 75.94% protein equivalent, was included among the collaborative samples to give some indication of the accuracy of the 2 methods. The mean of the copper catalyst results was 75.61% with a standard deviation of 0.45. The mean of the mercury catalyst results was 75.46% with a standard deviation of 0.49%. Three or 4 laboratories seem to be substantially lowering the mean in both methods. Because complete digestion is assured with this material, a check of apparatus, reagents, and technique is necessary if recovery is less than theoretical. A Kjeldahl room is probably a prime candidate for relaxed quality control in many laboratories. In any case, the copper method compares favorably with the mercury method with this sample.

Sample 47, Eastman lysine.HCl, 95.86% protein theoretical, was included because it is a hard-to-digest material and, therefore, a good test of the digestion parameters of the 2 methods. The purity of the material was given as "at least 98%." The mean of the copper catalyst results was 94.02% with a standard deviation of 1.34. The mean of the mercury catalyst results was 94.54% with a standard deviation of 1.50.

The copper catalyst had 3 considerably low values which the Dixon test had identified as outliers; these were, therefore, not included in the calculation of mean and standard deviation. It is plausible, however, that the cause of these

Table 4. Means and standard deviations, Cu vs Hg

Sample	Cu					Hg				
X–Y	Mean X	Mean Y	S_r	S_d	S_b	Mean X	Mean Y	S_r	S_d	S_b
1,28–11,29	18.757	16.868	0.198	0.286	0.206	18.749	16.858	0.276	0.380	0.261
2,23–7,37	87.661	88.890	0.668	1.04	0.796	87.658	88.888	0.529	1.08	0.945
3,39–15,27	15.701	16.155	0.256	0.319	0.190	15.715	16.191	0.263	0.279	0.094
4,26–17,43	54.208	53.843	0.361	0.611	0.493	54.093	53.732	0.445	0.632	0.449
5,54–25,51	13.369	10.121	0.146	0.238	0.188	13.465	10.084	0.182	0.232	0.144
6,44–10,33	29.758	29.272	0.302	0.446	0.328	29.715	29.227	0.390	0.499	0.312
8,46–14,36	26.963	28.097	0.217	0.391	0.326	26.885	27.992	0.193	0.433	0.387
9,38–13,42	85.786	90.104	0.723	1.06	0.780	85.713	90.043	0.842	1.14	0.772
16,45–19,41	39.419	40.432	0.644	0.801	0.477	39.424	49.350	0.495	0.677	0.462
18,30–34,40	84.916	80.708	0.667	0.835	0.503	85.091	80.683	0.570	0.967	0.780
12,35–21,49	17.010	17.937	0.195	0.236	0.133	17.019	17.923	0.460	0.528	0.258
22,50–31,52	17.750	17.788	0.169	0.242	0.172	17.681	17.859	0.194	0.290	0.216
24,53–32,48	44.016	44.408	0.562	0.604	0.223	44.136	44.625	0.328	0.484	0.355

Table 5. Approximate standard deviations by % protein level

% Protein range	Copper			Mercury		
	S_r	S_d	S_b	S_r	S_d	S_b
10–20	0.196	0.266	0.179	0.292	0.358	0.205
20–30	0.263	0.419	0.327	0.308	0.467	0.352
30–40	0.644	0.801	0.477	0.495	0.677	0.462
40–50	0.562	0.604	0.223	0.328	0.484	0.355
50–60	0.361	0.611	0.493	0.445	0.632	0.449
60–70	—	—	—	—	—	—
70–80	—	—	—	—	—	—
80–90	0.687	0.984	0.706	0.662	1.06	0.836

low values was incomplete digestion. Also, both mercury and copper standard deviations are high in comparison with typical standard deviations at the 80–90% protein level from Table 5.

These means and standard deviations are disappointing for both copper and mercury. Previous work has shown the copper digestion parameters to be rugged (4). Work in the author's and other laboratories indicates better performance could be expected.

To identify the sources of error in the method, the relation between heat input and lysine recovery was examined. Table 6 shows that there is some, but not consistent, correlation between the 2 factors. Evidently, a combination of digestion factors was affecting the data more than was noticed with the other sample materials.

It can be concluded that too many laboratories do not give enough attention to the various digestion parameters in either the copper or the mercury versions. The mercury method is sensitive to these parameters, and the copper method is somewhat more sensitive.

Analyses of variance, by method and laboratory, were run for each blind duplicate pair of feed samples. Of the 26 pairs, 22 showed no method effect and 4 did show an effect at the 95% confidence level (Table 7). Of the 4 showing a method effect—the 2 meat and bone meals, the puppy food, and a milk replacer—the grand mean for copper was higher than that for mercury in each case.

Conclusions and Recommendations

On a wide variety of sample types, the copper catalyst version of the method performed at least as well as the mercury catalyst version. Means were either equivalent or slightly higher with copper than mercury. Standard deviations were equivalent or slightly lower with copper than mercury, and one sample had a noticeably higher standard deviation with mercury. In the few sample sets where there was a statistically significant difference between the methods, copper means were slightly higher than mercury means. The improvement in copper catalyst performance over previous studies is attributed to an optimization of the digestion parameters for copper. Performance on NBS $NH_4H_2PO_4$ was slightly better with the copper catalyst than with the mercury catalyst.

The only negative factor in the study was the performance of the copper method with lysine.HCl. While both methods gave high standard deviations with this material, evidence suggests that with especially difficult-to-digest material, the copper digestion parameters are less rugged than the mercury parameters.

It is recommended that the copper catalyst method be adopted official first action; that it be stipulated as a part of the method that lysine.HCl be analyzed during routine sample determinations as an ongoing check of analytical performance; that the question of the indefinite titration end point of lysine noted by one collaborator be further investigated; that if bumping is a problem during the distillation step, the volume of dilution water be increased from 250 to 275 mL.

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Table 6. Relation between boil test and lysine digestion

Lab.	Boil test	Cu result, lysine	Hg result, lysine
1	5–6	—	92.54
2	5½–7	95.15	95.36
3	7–9	86.50	93.50
4	5–8	85.38	96.85
5	8 av.	92.04	92.04
6	5–7	95.58	95.00
7	4½–5	95.17	95.38
8	4½–5	93.56	93.01
9	4–5	91.50	94.50
10	not properly detd	94.44	95.74
11	9–13	93.99	94.68
12	5	93.75	93.94
13	4–6½	92.35	91.59
14	6–8	89.70	94.50
15	4–5½	95.47	95.40
16	4–11	95.75	96.65
17	4–5½	94.77	95.78
18	6–9	95.12	95.66
19	5–6½	92.09	92.58
20	<8	90.93	94.08
21	4½–5	94.6	95.80
22	3½–4	94.76	95.82
22[a]	7	94.64	95.31

[a]Lab. 22 ran 2 sets of results, one with 750 watt heaters and one with 500 watt heaters, on equipment that did not permit heat adjustments.

Table 7. Analyses of variance and grand means

Sample	Significant method effect?	Copper grand mean	Mercury grand mean
1–28	no	18.75	18.75
2–23	no	87.66	87.60
3–39	no	15.70	15.72
4–26	yes	54.20	54.02
5–54	no	13.36	13.46
6–44	no	29.76	29.72
7–37	no	88.89	88.89
8–46	no	26.96	26.88
9–38	no	85.79	85.71
10–33	no	29.27	29.24
11–29	no	16.85	16.86
12–35	no	17.00	17.02
13–42	no	90.18	90.06
14–36	yes	28.10	27.99
15–27	no	16.13	16.17
16–45	no	39.42	39.42
17–43	yes	53.84	53.63
18–30	no	84.82	84.94
19–41	no	40.40	40.32
21–49	no	17.94	17.92
22–50	yes	17.75	17.67
24–53	no	44.02	44.14
25–51	no	10.12	10.08
31–52	no	17.79	17.86
32–48	no	44.41	44.63
34–40	no	80.71	80.68

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Simple, Rapid Method for Determination of Total Extractable Fat in Canned Pet Foods

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The rapid column method described, unlike AOAC method 7.056, determines both neutral ("crude") and *total* fat in canned pet foods, and uses nonflammable solvent mixtures and simple laboratory equipment. Neutral fat values are obtained by eluting the column with dichloromethane, whereas *total* fat values are determined by using dichloromethane–methanol (9 + 1). For 7 samples analyzed in triplicate, fat ranged from 2.9 to 10.8%. Neutral fat values by the dry column method were significantly lower ($P < 0.05$) than were those by 7.056 (6.29 vs 6.49), although these differences were practically unimportant. *Total* fat determinations by the dry column method and by 7.056 yielded overall means of 7.40 and 6.49%, respectively. The 0.91% mean difference is significant ($P < 0.01$) and represents a more complete extraction of polar lipids by the proposed method.

Determination of fat content of canned pet foods by the AOAC Soxhlet method **7.056** (1) requires several hours for a complete analysis, uses flammable solvents which require fume hoods and other safety equipment, and produces a "crude" or ether-soluble extract for the fat value. Moreover, crude fat values may be misleading as indicators of fat content because they do not fully represent the potentially large amounts of phosphclipids in such foods.

A method of fat extraction previously developed in this laboratory for meat and meat products (2), which alleviates many problems encountered with Soxhlet determinations, has now been modified for analysis of canned pet foods. By use of this simple, dry column procedure, either neutral (crude) or total fat values may be obtained, and the unaltered fats recovered by this method may be analyzed further (3).

METHOD

Reagents and Apparatus

Dichloromethane (DCM) and methanol were obtained from Burdick and Jackson Laboratories (Muskegan, MI 49442). Column packings were granular anhydrous Na_2SO_4 (Mallinckrodt, Inc., Paris, KY 40361); $CaHPO_4 \cdot 2H_2O$; and Celite 545, not acid-washed, Catalog No. C-212 (Fisher Scientific Co., King of Prussia, PA 19406). Celite 545 and $CaHPO_4 \cdot 2H_2O$ (9 + 1) were mixed and then stored in a covered glass jar until needed.

A porcelain mortar (750 mL) and pestle (2), a glass chromatographic column ca 25 mm id × 30 cm long with a drip tip 5 cm × 8 mm id (prepared locally), and a tamping rod were used.

Preparation of Sample for Analysis

Samples were heated in their cans for 30 min in a 45°C water bath. The cans then were opened, and their contents were mixed in a food processor until uniform. The mixture was reheated at 45°C to ensure uniformity, and then samples for both **7.056** and dry column methods were removed and weighed on disposable tared aluminum weighing pans (use smooth-wall pans).

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Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Procedure

(1) Place glass wool plug loosely into tip of glass column, charge column with 5 g previously prepared Celite 545–$CaHPO_4$ mixture, and tamp firmly in place. Place preweighed container (150 mL tall-form beaker) under drip tip.

(2) Weigh a sample of canned food (ca 2.5 g) to nearest 0.1 mg and transfer quantitatively to mortar. (Use spatula to transfer bulk of sample, then some Na_2SO_4 from step *3* to pick up remaining sample from weighing dish.)

(3) Add 10 g anhydrous Na_2SO_4 to mortar and mix thoroughly using pestle. Then add ca 7 g Celite 545 and grind until a uniform free-flowing powder is formed (ca 2 min).

(4) Transfer resultant mixture to glass column through powder funnel and firmly tamp in place.

(5) Neutral (crude) fat determination.—Rinse mortar with ca 25 mL DCM by use of a large disposable pipet and transfer rapidly to inner column wall. Let solvent pass through column bed until a first drop of eluant appears at drip tip, then immediately add additional 100 mL DCM and let column drip until dry. Collected solvent may be removed during elution by gentle heating and evaporation under nitrogen stream. If fat is required for further analytical studies, eluate may be collected in volumetric flask instead of beaker.

(6) Total fat determination.—Follow step *5* except use solvent mixture of DCM–methanol (9 + 1) to wet the column and then use an additional 100 mL to charge column.

(7) After solvent removal from either steps *5* or *6*, dry residue in beaker for 30 min at 100°C as specified in sec. **7.056** (1). Determine residue weight and calculate percent (%) fat as (residue wt/sample wt) × 100.

Results and Discussion

Although the dry column extraction method was previously applied only to meat and meat products (2), no problems were experienced when the method was attempted with canned pet foods. The present work used half the sample size and reagents used in the previous study with no effect on accuracy, and this resulted in a considerable saving in reagents. No nonlipid artifacts were found in the fat extracts, indicating that the $CaHPO_4$–Celite 545 trap was effective in retaining nonlipid materials of canned foods. Total elapsed time required to complete an analysis by the dry column method is typically 1.5–2 h including the 30-min drying time needed for the fat residue, which compares favorably with that for a standard AOAC Soxhlet method **7.056** (6–8 h including sample drying time). In addition, the dry column method uses less hazardous solvents, dichloromethane and methanol, rather than flammable ethers.

AOAC method **7.056** measures crude fat, the composition of which may vary with the ether used as solvent. Small but inconsistent amounts of phospholipid are co-extracted with the neutral fat when using ethyl but not petroleum ether (4). The present study confirmed this observation where the phospholipid content of each of the ethyl ether Soxhlet extracts was measured. Each sample contained low levels (0.05–0.50%) of phospholipid (Table 1), suggesting that values obtained for fat content by Soxhlet do not represent a clearly defined portion of total fat in canned processed foods. In contrast, by proper choice of solvent with the column method, one can

Table 1. Comparison of AOAC 7.056 and dry column methods for extraction of crude and total fat and phospholipids from pet foods

		Dry column			AOAC Soxhlet[a]	
Supplier	Type	Total fat,[b,c] %	PL[b,d] %	Neutral fat,[b,e] %	Crude fat,[b] %	PL,[b,d] %
	Dog foods:					
A	Beef	9.60 ± 0.14	0.76	8.52 ± 0.22	8.65 ± 0.15	0.48
B	Chicken	7.80 ± 0.08	0.25	7.05 ± 0.05	7.03 ± 0.06	0.05
A	Lamb	5.18 ± 0.01	0.73	4.32 ± 0.13	4.50 ± 0.25	0.32
A	Liver	6.41 ± 0.06	0.99	5.33 ± 0.03	5.63 ± 0.06	0.48
	Cat foods:					
C	Beef and liver	10.83 ± 0.08	1.37	9.33 ± 0.05	10.05 ± 0.38	0.50
D	Salmon	9.08 ± 0.12	1.43	7.58 ± 0.05	7.58 ± 0.16	0.46
E	Tuna	2.93 ± 0.03	0.89	1.91 ± 0.03	1.99 ± 0.28	0.13
Mean of 7 samples		7.40 ± 0.07	0.92	6.29 ± 0.08	6.49 ± 0.19	0.35

[a]AOAC reference method **7.056** (1).
[b]Mean of 3 subsamples ± std dev.
[c]Column eluted with DCM–methanol (9 + 1).
[d]Phosphorus content determined by method of Vaskovsky et al. (5), and expressed as % phospholipid (PL) = 25 × % phosphorus.
[e]Column eluted with DCM only.

accurately determine either the neutral or total fat in a canned food sample. When dichloromethane is used as the sole eluant, only the neutral fat is removed from the sample (3); therefore, no values for recovered phospholipid are shown in Table 1. For the reasons stated, direct comparisons between crude (Soxhlet) and neutral fat values (dry column method) cannot be made. Their values are similar (6.29 vs 6.49%), however, and although the neutral values are significantly lower ($P < 0.05$), the absolute difference between the means is only 3.1%.

The major utility of the dry column method is its ability to obtain values for the *total* fat content of canned foods. Earlier experiments with meat (3) demonstrated that *total* fat was recovered when the dry column was eluted with DCM–methanol (9 + 1). Similar results were obtained in the present work with canned pet foods, where the values for total fat (7.40 vs 6.49%) were significantly ($P < 0.01$) higher than those determined by **7.056** (Table 1). The mean difference (0.91%) between these values reflects, in part, the amounts of additional phospholipid extracted by the proposed method. Moreover, fat recovered by the dry column method is not altered by the extraction process (3) and may be used in subsequent analytical studies. Thus, the dry column fat extraction method affords the analyst the option of attaining either the neutral (crude) or total fat content of canned pet foods by techniques that are simple to perform. In limited tests with canned processed foodstuffs other than pet foods, the proposed method worked equally well and could be considered as an alternative when these substances are analyzed for fat content.

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FOOD ADDITIVES

Extraction of Organic Acids by Ion-Pair Formation with Tri-*n*-Octylamine. Part V. Simultaneous Determination of Synthetic Dyes, Benzoic Acid, Sorbic Acid, and Saccharin in Soft Drinks and Lemonade Syrups

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Synthetic dyes, benzoic acid, sorbic acid, and saccharin are extracted simultaneously from soft drinks with 0.01M tri-*n*-octylamine at pH = 5.5 and are back-extracted to an aqueous phase with 0.1M sodium perchlorate. The perchlorate solution is injected directly into the reverse phase liquid chromatographic system which permits the separation of all the substances investigated. Forty-six commercial samples of soft drinks and 11 lemonade syrups were analyzed. All samples conformed to the legal prescriptions.

The Belgian food legislation, as well as others, regulates the use of food additives. In addition to the generally applied principle of positive lists, Belgian legislation has also prescribed the maximum amount allowed for a given additive in a given foodstuff. The legislation on food additives contains a chapter for each group of foods and enumerates the various permitted additives. An analytical method which would enable the simultaneous determination of different classes of additives should be of great value for the routine control of victuals.

As described in previous papers (1, 2), food dyes are quantitatively extracted by ion-pair formation with tri-*n*-octylamine (TnOA). Identification and determination are possible by direct chromatography of the extract or after a back-extraction to an aqueous phase (3). Both thin layer chromatography (TLC) (3) and ion-pair liquid chromatography (LC) (3, 4) have been used. Many authors have described the determination of preservatives and saccharin by LC. Some papers describe the quantitation of sorbic acid (5, 6), mixtures of benzoic acid and sorbic acid (7, 8), saccharin (9), or mixtures of all or some of these compounds (10–12). Various cleanup procedures such as direct injection after filtration (5, 8, 11), steam distillation (5, 7), acid extraction (9), or Extrelut cleanup columns (12) were used.

In this paper, the applicability of the TnOA extraction scheme, as it was used for dyes, is reported for preservatives and saccharin. It was applied to the analysis of commercial soft drinks and their concentrates. A subsequent paper will describe the determination of the same additives in a more complex foodstuff, e.g., yogurt.

Experimental

Apparatus and Reagents

(a) *Chromatograph.*—Varian LC 5060, equipped with manual Valco loop injector (loop size 100 μL), standard Varian 254/280 nm UV detector (eluates were monitored at 254 nm), and 250 × 4 mm id RP-18 (Lichrosorb) column from Merck, 10 μm octadecyl silica. Integrations were done using a Varian Vista CDS 401.

(b) *pH meter.*—Orion Ionalyzer 601 and combined glass electrode.

(c) *Spectrophotometer.*—Perkin Elmer Hitachi 200 with 10 mm quartz cells.

(d) *Ultrasonic bath.*—Branson Model B32.

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(e) *Mobile phase.*—Mobile phases were prepared by LC system. The apparatus was equipped with 2 bottles: The first contained methanol and the second contained buffer. Buffers for LC were filtered through a 0.45 μm (Millipore) filter.

(f) *Standards.*—Dyes were obtained from P. Entrop (Machelen, Belgium). Potassium sorbate, methyl-*p*-hydroxybenzoate (commonly referred to as nipagin), ethyl-*p*-hydroxybenzoate, and propyl-*p*-hydroxybenzoate (also referred to as nipazol) were obtained from Fluka (Buchs, Switzerland). Sodium saccharin was obtained from B.D.H. (Poole, England); sodium benzoate, citric acid, and caffeine were obtained from Merck (Darmstadt, GFR).

(g) *Stock solutions.*—*Stock solution A:* 1 g sodium benzoate/L; *stock solution B:* 1 g potassium sorbate/L; *stock solution C:* 1 g sodium saccharin/L. All stock solutions were prepared in double distilled water; dilutions were made in phosphate buffer (pH = 5.5; ionic strength = 0.1).

(h) *Tri-n-octylamine.*—Aldrich Europe (Beerse, Belgium).

(i) *Buffers.*—Composition for 2 L and ionic strength 0.1:

pH = 7.0	5.780 g $NaH_2PO_4.H_2O$	9.380 g $Na_2HPO_4.2H_2O$
pH = 5.5	24.650 g $NaH_2PO_4.H_2O$	1.260 g $Na_2HPO_4.2H_2O$
pH = 4.5	0.9 mL 1M H_3PO_4	27.598 g $NaH_2PO_4.H_2O$
pH = 4.0	3.2 mL 1M H_3PO_4	27.598 g $NaH_2PO_4.H_2O$
pH = 3.0	32.0 mL 1M H_3PO_4	27.598 g $NaH_2PO_4.H_2O$

(j) *Solvents.*—Methanol was LC grade from Merck; all other reagents and solvents were analytical grade from Merck.

(k) *Cellulose TLC plates.*—Layer thickness 0.1 mm (Merck).

Extractions

(a) *From aqueous solutions.*—Ten mL 10 mg/L solution of the analyte, in phosphate buffer with pH 5.5 and ionic strength 0.1, was shaken 30 min with 10 mL 0.1 or 0.01M TnOA solution in chloroform in a stoppered 30 mL centrifuge tube. After phase separation by centrifugation, 7 mL chloroform layer was transferred to a second centrifuge tube and shaken 30 min with 7 mL 0.1M sodium perchlorate. Concentration of analyte in the aqueous phase was measured by photometry.

All extractions were performed with aqueous and organic phases which had previously been saturated with one another by shaking equal volumes (ca 400 mL) of phosphate buffer (pH = 5.5; ionic strength = 0.1) and 0.01M TnOA solution in chloroform for 10 min at room temperature.

(b) *From spiked samples.*—*(1) Spiking of soft drinks.*—Benzoate: 1, 5, and 10 mL stock solution A were diluted to 100 mL with blank soft drink; sorbate: 1, 2.5, and 5 mL stock solution B were diluted to 100 mL with blank soft drink; saccharin: 1, 2.5, 5, and 10 mL stock solution C were diluted to 100 mL with a blank soft drink.

(2) Extraction.—Lemonade syrups: 10 g was diluted with water to 100 mL. Soft drinks were degassed prior to analysis by ultrasonication (15 min at room temperature). Five mL degassed soft drink or diluted lemonade syrup was mixed in a centrifuge tube with 5 mL phosphate buffer (pH = 5.5 and ionic strength 0.5); the pH was controlled and, if necessary,

Table 1. Retention times as function of mobile phase methanol content[a]

	Methanol content					
	Ionic strength = 0.1		Ionic strength = 0.01			
Compound	35%	30%	50%	40%	35%	30%
Benzoic acid	4.81	6.18	2.92	3.50	4.16	5.09
Sorbic acid	5.83	8.06	3.15	3.91	4.67	5.98
Saccharin	4.93	7.28	2.80	3.58	4.50	6.24
Tartrazine	9.80	30.46	2.00	3.53	4.57	7.17
Nipagin	14.0					
Nipazol	30		5.27	3.40	17.09	24.02
Caffeine				4.02	5.80	5.15

[a]Methanol–phosphate buffer, pH 7.0, containing 0.005M tetrabutylammonium phosphate.

adjusted to 5.5 ± 0.1 with 2M sodium hydroxide. The extraction was performed with 10 mL 0.01M TnOA solution in chloroform. After phase separation, 7 mL organic phase was transferred to a second tube and extracted with 7 mL 0.1M sodium perchlorate. The aqueous phase was injected into the LC system for analysis.

Analysis of Commercial Samples

(a) *Extraction.*—Commercial samples were extracted by the procedure given above for spiked samples.

(b) *Identification.*—Extracted analytes were identified by their retention times, dyes were identified also by TLC on cellulose TLC plates with ethyl acetate–*n*-propanol–ammonia–water (35 + 35 + 20 + 20) as mobile phase. This system was described earlier (2, 3).

(c) *Calibration.*—Calibration curves were constructed by plotting area of the LC peak vs concentration of the standards (0–50 mg/L). Amount of additive in the extracts was determined by a curve-fitting program. The following calibration curves were obtained:

sorbic acid:
peak area = 253122 × concentration − 2605
benzoic acid:
peak area = 9453 × concentration − 1236
saccharin:
peak area = 5930 × concentration − 1167

The correlation coefficients were 0.9997 or higher. The between-day variation of the slopes was less than 2% for the 3 compounds investigated ($N = 4$).

Results and Discussion

Separation of Food Additives by LC

A reverse phase system was chosen with the aim of simultaneously determining as many additives as possible. Several papers describe the determination of benzoic acid and sorbic acid (5–8), saccharin (9), or mixtures of these compounds (10–12) by reverse phase LC. Conditions for optimization of the LC system for dye analysis were chosen as the starting point. Dyes were separated on an RP-18 column, the mobile phase was composed of a mixture of methanol and phosphate buffer with pH = 7 (2–4), and 0.005M tetrabutylammonium was added to the mobile phase to increase retention of the hydrophilic solutes. Table 1 gives results of the experiments done with this system. Even with methanol concentrations as low as 30%, no satisfactory separations can be obtained for benzoic acid, sorbic acid, and saccharin. The use of a more dilute buffer, which should increase the effect of the counter ion (13), results in lower retention times but with no increased selectivity (Table 1). It is, however, interesting to note that the more dilute buffer results in a different elution order. Use of the more dilute buffer is especially convenient for the chromatography of *p*-hydroxybenzoic acid esters (nipagin and nipazol) and caffeine because their retention is markedly decreased.

Because the separations at pH = 7 were not successful, the pH of the mobile phase was decreased and the counter ion was left out. Attention was then focused on the separation of benzoic acid, sorbic acid, saccharin, and tartrazine. Tartrazine was chosen because it is one of the early-eluting dyes. The retention times of a few other additives are given as well. Benzoic acid and sorbic acid are not resolved at pH 3.0, nor are they resolved with 20% or 50% methanol (Table 2). The acid dissociation constant of benzoic acid is 4.2, and that of sorbic acid, 4.7 (14). Both acids are highly undissociated at pH 3.0, and the compounds are not separated. This means that the undissociated acids have an equal distribution constant between the mobile phase and the hydrophobic chromatographic support. Furthermore, the fully dissociated acids, which are formed at a pH of 7, are not separated either. Therefore, the separation was then tried at intermediate pH values so that the acids are partially dissociated. Table 2 gives the results of experiments carried out at pH 4.0 and 4.5. The data indicate that, with a methanol concentration of 30%, satisfactory separations can be obtained both at pH = 4.0 and 4.5. At pH 4.5, the separation is achieved in a shorter time; this is important for additives like caffeine and nipazol which have rather high retention times. Under these pH conditions, the separation of benzoic acid, sorbic acid, saccharin, and tartrazine is also quite successful. All further analyses were carried out with mobile phases composed of a phosphate buffer of pH 4.5.

The combination of the RP-18 column with an eluant composed of methanol and a pH 4.5 phosphate buffer is very powerful. Various groups of additives can be separated. Figure 1 shows the separation of synthetic dyes (tartrazine, amaranth, sunset yellow, and cochenille red), preservatives (citric acid, benzoic acid, sorbic acid, and methyl, ethyl, and propyl esters of *p*-hydroxybenzoic acid), and flavorings (saccharin and caffeine). To obtain a satisfactory separation in an acceptable time, however, a methanol gradient is required.

Extraction

A former paper (15) demonstrated that it is preferable to extract with 0.01M TnOA instead of 0.1M where extraction yields are less than 100%. Table 3 shows that back-extraction with 0.1M sodium perchlorate is more efficient when lower TnOA concentrations are used, as it provides higher overall yield. When different concentrations of sorbate, benzoate, and saccharin were extracted, it was observed that the extraction yield was higher with higher analyte concentrations (Table 4). This is especially evident for sorbic acid and saccharin but less so for benzoic acid. The mean extraction recovery was calculated for quantitative analysis, and the results were

Table 2. Retention times (min) as function of pH and mobile phase methanol content[a]

	Methanol content					
	pH = 3.0		pH = 4.0		pH = 4.5	
Compound	50%	20%	50%	30%	50%	30%
Benzoic acid	4.51	20.0	3.79	7.84	3.24	6.43
Sorbic acid	4.61	20.0	4.08	9.84	3.77	8.54
Saccharin	2.25	3.68	4.29			3.55
Tartrazine	2.26	4.65	1.95			2.47
Nipagin	4.43					
Nipazol	9.23					
Caffeine	3.93		3.68			9.34

[a]Methanol–phosphate buffer (ionic strength 0.1).

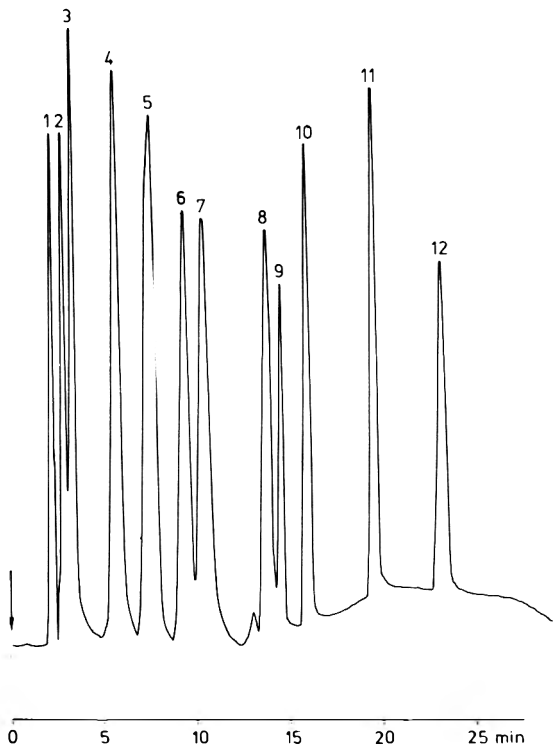

Figure 1. Separation of various food additives

Gradients listed under *Apparatus and Reagents*. (1) citric acid, (2) saccharin, (3) tartrazine, (4) amaranth, (5) benzoic acid, (6) sunset yellow, (7) cochenille red, (8) sorb c acid, (9) caffeine, (10) *p*-hydroxybenzoic acid methyl ester, (11) *p*-hyd·oxybenzoic acid ethyl ester, (12) *p*-hydroxybenzoic acid propyl ester.

Gradient profile: t = C min: 30% methanol; t = 5 min: 30% methanol; t = 15 min: 60% methanol; t = 22 min: 60% methanol; t = 30 min: 30% methanol.

Table 3. Overall extraction yield (%) from buffered aqueous solutions: extraction with different TnOA concentrations, back-extraction with 0.1M sodium perchlorate (initial concentration: 50 mg/L)[a]

	TnOA concentration	
Compound	0.1M	0.01M
Benzoic acid	65.5 ± 1.6	78.0 ± 2.6
Sorbic acid	68.5 ± 1.4	81.5 ± 3.0
Saccharin	23.2 ± 0.1	71.0 ± 3.0

[a]N = 3.

Table 4. Extraction (%) from spiked lemonades: extraction with 0.01M TnOA, back-extraction with 0.1M sodium perchlorate[a]

Concn, mg/L	Benzoic acid	Sorbic acid	Saccharin
10	77.9 ± 4.1	65.6 ± 1.1	60.6 ± 1.3
25	—	68.1 ± 0.9	68.1 ± 1.9
50	78.2 ± 2.6	81.7 ± 0.8	70.1 ± 3.6
100	82.8 ± 3.3	—	71.0 ± 3.4

[a]N = 3.

multiplied by the following factors: benzoate 1.25, sorbate 1.40, and saccharin 1.50.

The increase in extraction yield as a function of analyte concentration might be due to the occurrence of side reactions such as the formation of adducts or aggregates. Benzoic and sorbic acids (both at the 25 mg/L level) were extracted alone and together to investigate whether the co-extraction of one acid influences the yield obtained for the other. The recoveries (N = 6) were sorbate alone 65.1% (± 2.5), sorbate in a mixture with benzoate 65.3% (± 1.8), benzoate alone 80.2% (± 3.0), and benzoate with sorbate 80.3% (± 3.7). These data indicate that recoveries of either acid were not influenced by the presence of the other.

Table 4 shows data for extraction of spiked soft drinks. At the 50 mg/L level, recoveries were equal to those for aqueous solutions. It is essential to control the pH of the extracting medium. In fact, some very acidic soft drinks were not buffered at pH 5.5 even with the concentrated buffer (ionic strength 0.5). When the pH is lower than 5.5, ion-pair formation is reduced in favor of the liquid-liquid distribution of the acid analytes. This results in a lower extraction yield but, above all, in an inefficient back-extraction with perchlorate.

With this experimental scheme, the detection limits, given by the lowest concentration that was still detected as a peak by the integrator (signal-to-noise ratio = 2, 0.01 AUFS), were 0.1 mg/L for benzoic acid and saccharin. For sorbic acid, the determination limit is much lower, i.e., 0.005 mg/L, than that for benzoic acid and saccharin because of the higher specific absorption of sorbic acid.

Other additives such as nipagin, nipazol, and caffeine are also extracted quantitatively from buffered solutions with 0.1M TnOA. At pH = 5.5, neither of these compounds is present as an anion, which indicates that their extraction is based on a simple liquid-liquid distribution. Consequently, they cannot be back-extracted with perchlorate, and they should be determined directly in the chloroform phase (not attempted yet). Earlier papers (3, 16) showed that it is possible to identify analytes (extracted as ion pairs with TnOA) directly in the chloroform extract by evaporating the chloroform and redissolving the residue in ethanol, which is then injected into the LC system. The identification is then based on the comparison of retention times of the unknown substances (injected as ion pairs) with standards injected as aqueous solutions. It was observed, however, that the repeated injection of such extracts causes a gradual peak distortion due to the adsorption of TnOA by the hydrophobic stationary phase. Rinsing the column regularly with ethanol restores the initial separation efficiency.

Table 5. Results for commercial soft drinks

Soft drink	No. of samples	No. free of additives	Amounts, mg/L[a] Sorbic acid	Benzoic acid	Saccharin	Dyes[b]
Orange	14	7	20	—	—	—
			—	10	—	—
			—	30	—	—
			—	40	—	—
			10	30	—	—
			10	—	—	E102 + E110
			—	—	—	E102 + E110
Lemon	14	7	20	—	—	—
			30	—	—	—
			—	20	—	—
			—	30	—	—
			—	50	—	—
			10	—	—	E102 + E110
			10	—	35	E102
Cola	6	6	—	—	—	
Various	12	8	90	—	—	—
			—	40	—	—
			—	80	—	—
			—	—	—	E124
Maximum levels (mg/L)			100	100	75	

[a](—) Not detectable.
[b]E102 = tartrazine = FD&C Yellow No. 5 = C.I. No. 19140.
E110 = sunset yellow = FD&C Yellow No. 6 = C.I. No. 15985.
E124 = cochenille red = C.I. No. 16255 = ponceau 4R.

Table 6. Results for commercial lemonade syrups (mg/kg)[a]

Fruit syrup	Manuf.	Sorbic acid	Benzoic acid	Saccharin	Dyes
Strawberries	A	—	—	—	—
	B	25	—	—	—
Red berries	B	36	134	—	—
Blackberries	A	—	—	—	—
	B	40	—	—	—
Orange	B	30	—	—	—
Lemon	B	20	60	—	—
	C	20	80	—	—
Apple	C	48	60	—	—
Grapefruit	A	20	—	—	—
Anise	A	—	—	—	—
Maximum levels (mg/kg)		250	250	0	500[b]

[a](—) Not detectable.
[b]Total of synthetic and natural dyes; specific dyes have their own maximum, e.g., tartrazine 100 mg/kg.

Analysis of Samples

All samples were analyzed following the same methodology. Although some samples could have been analyzed by direct injection into the LC system, they were also extracted to protect the analytical column. Furthermore, the same method will also be tested for the analysis of more complex, solid foods.

Forty-four soft drink samples and 11 lemonade syrups were analyzed. The following additives were determined: synthetic dyes, benzoic acid, sorbic acid, and saccharin. Dyes were identified but not quantitated because their concentrations are not limited in Belgium by legal prescriptions.

Fourteen samples of orange soft drinks were analyzed; 7 of these contained no additives. Table 5 gives results for the other 7 samples. Three samples contained sorbic acid (10–20 mg/L), one of these contained a mixture of benzoic acid (30 mg/L) and sorbic acid (10 mg/L). Only 2 orange soft drinks contained synthetic dyes; in both cases, this was a mixture of tartrazine (E102, FD&C Yellow No. 5) and sunset yellow (E110, FD&C Yellow No. 6). One of these samples also contained 10 mg/L of sorbic acid.

Fourteen samples of lemon soft drinks were also analyzed; 7 of these were devoid of additives (Table 5). Benzoic acid was found in 3 samples (20–50 mg/L), and sorbic acid was present in 2 others (20 and 30 mg/L). One sample contained 10 mg/L of sorbic acid as well as tartrazine and sunset yellow. The orange and lemon samples which contained sorbic acid + tartrazine + sunset yellow were from the same manufacturer. Only one soft drink sample contained saccharin (35 mg/L), together with sorbic acid (10 mg/L) and tartrazine.

Six cola samples, all from different manufacturers, were all free of preservatives and saccharin (Table 5). In 12 other soft drink samples, 2 contained benzoic acid (40 and 80 mg/L), and another contained 90 mg/L of sorbic acid. Only one sample (a grenadine) was colored; cochenille red was found. Therefore, 28 of the 46 soft drink samples contained none of the additives investigated, and all other samples were under the imposed maximum concentrations. Figure 2 shows chromatograms from 3 soft drinks. The retention times (min) of the various additives in the system used (20% methanol) are citric acid 2.0; saccharin 3.5; tartrazine 4.9; amaranth (E123, formerly FD&C Red No. 2) 7.8; benzoic acid 8.5; sunset yellow 9.5; cochenille red A (E124, C.I. 16255) 12.1; and sorbic acid 13.9.

Eleven different commercial lemonade syrups were analyzed; Table 6 gives the results. Only 3 samples, all from the

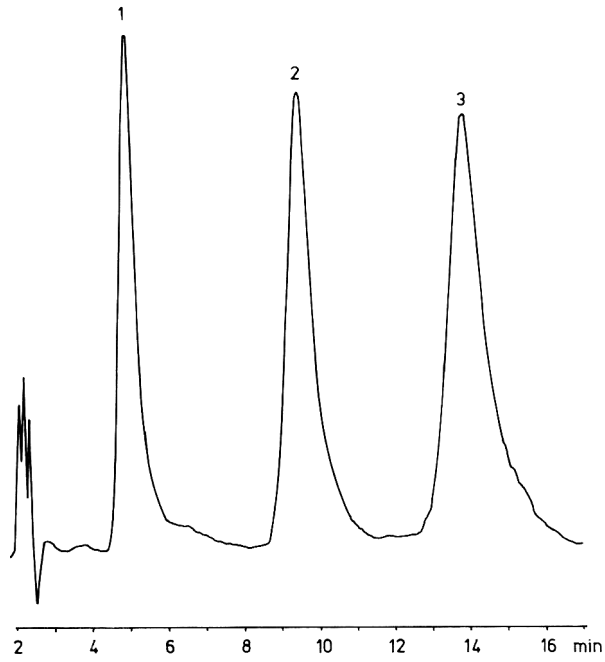

Fig. 2a

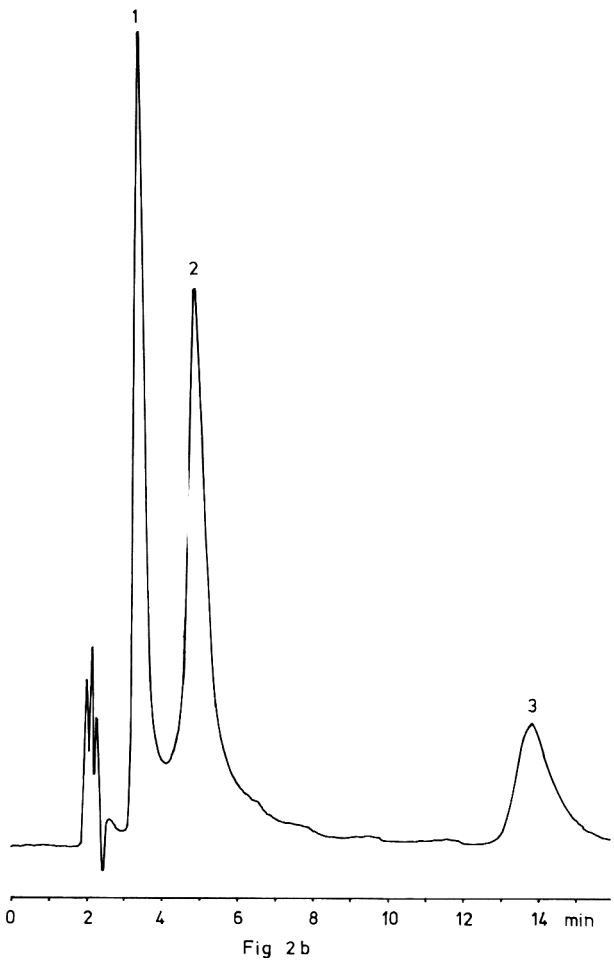

Fig 2b

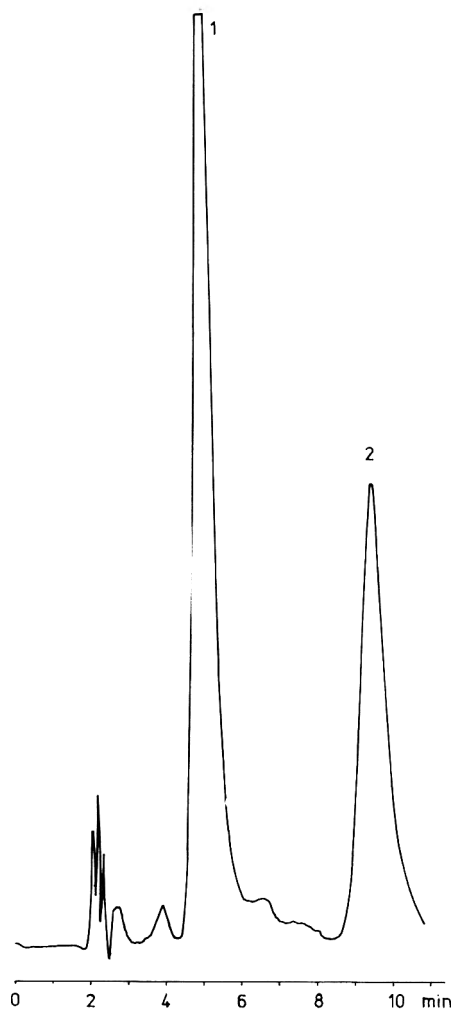

Fig. 2c

Figure 2. Chromatograms obtained for soft drink extracts (mobile phase: methanol–phosphate buffer pH = 4.5 (20 + 80)).

2a: (1) tartrazine, (2) sunset yellow, (3) sorbic acid; 2b: (1) saccharin, (2) tartrazine, (3) sorbic acid; 2c: (1) tartrazine, (2) sunset yellow.

same manufacturer, were free of additives. All 5 samples from another manufacturer contained sorbic acid or a mixture of sorbic and benzoic acids. All samples complied with the legislation for additives.

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Liquid Chromatographic Determination of Acetic Acid in Foods

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A liquid chromatographic (LC) method has been developed for the determination of acetic acid in vinegar and other foods. The LC system includes an Aminex HPX-87H column and a UV detector set at 210 nm. The mobile phase is 0.009N H_2SO_4 at a flow rate of 0.7 mL/min. The method is simple and specific for acetic acid. Recoveries of acetic acid from a variety of products ranged from 93.3 to 102% with coefficients of variation from 2.4 to 4.6%.

Acetic acid is one of the earliest used and most common food acidulants. It is the major organic acid in vinegars and is among the organic acids present in many naturally fermented foods. Acetic acid, in the form of vinegar, is used in manufacturing many food products such as sour and sweet pickles, mayonnaise and salad dressings, mustards, various sauces, and ketchups. The quantitative determination of acetic acid in such food products is important because the acid content affects the quality and conformity to food regulations.

The present methods for the determination of acetic acid in foods involve distillation and titration with a base (1), gas chromatography of the derivatized acid (2), or liquid chromatography (LC) (3, 4). However, these methods either lack specificity or require a number of sample preparation steps. The main objective of this study was to develop a simple, specific, and versatile LC method for the quantitative determination of acetic acid in food products.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Waters Associates, equipped with Model 6000A pump, Model U6K injector, and data module (Waters Associates, Milford, MA 01757).

(b) *Detector.*—Gilson Model 222 (Gilson Medical Electronics, Middleton, WI 53562), set at 210 nm, and 0.10 sensitivity unit.

(c) *LC column.*—300 × 7.8 mm Aminex HPX-87H with Micro-Guard ion exclusion cartridge (Bio-Rad Laboratories, Richmond, CA 94804).

(d) *Centrifuge.*—Sorvall Model RC-5 with rotor No. SS-34 (DuPont Co., Newton, CT 06470).

(e) *Blender.*— Waring.

Reagents

(a) *LC water.*—Pass distilled, deionized water through 0.45 μm filter membrane.

(b) *LC mobile phase.*—0.009N H_2SO_4. Prepare in LC water.

(c) *Disodium ethylenediaminetetraacetate (EDTA) solution.*—1%. Dissolve 1 g EDTA (ACS, Fisher Scientific Co.) in 100 mL LC water.

(d) *Acetic acid stock solution.*—Pipet 5 mL concentrated glacial acetic acid into a 100 mL volumetric flask and dilute to volume with LC water. Titrate a 10 mL aliquot of solution with standardized 1N NaOH, using phenolphthalein as indicator. Calculate concentration of acetic acid in solution (mg/mL).

(e) *Acetic acid–EDTA standard solution.*—Pipet 5 mL of solution (d) into 100 mL volumetric flask, add 5 mL EDTA solution, and dilute to volume with LC water.

Sample Preparation

(a) *Liquids.*—Filter through Whatman No. 1 paper if necessary. Pipet proper volume (2 mL vinegar, 15 mL pickles brine) into 50 mL volumetric flask, add 2.5 mL EDTA solution, and dilute to volume with LC water. Refrigerate until LC analysis.

(b) *Solids and semisolids.*—Blend appropriate weight (10–20 g) with 50 mL water for 3 min. Filter or centrifuge-blend at 10 000 rpm for 10 min. Wash sediment with 10 mL water, recentrifuge, and combine wash with supernate. Transfer filtrate or supernate to 100 mL volumetric flask, add 5 mL EDTA solution, and dilute to volume with LC water. Refrigerate until LC analysis.

LC Analysis

Set flow rate at 0.7 mL/min and inject 10 μL acetic acid–EDTA standard solution into liquid chromatograph. Establish retention time and peak area and calibrate data module

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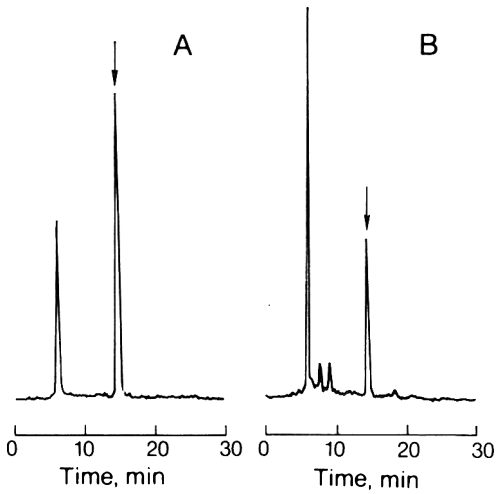

Figure 1. Chromatograms of distilled white vinegar (A) and mayonnaise (B). Arrow shows acetic acid peak. First peak in both A and B is EDTA.

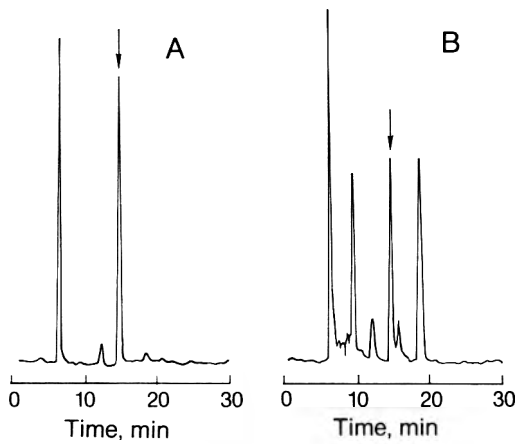

Figure 2. Chromatograms of dill cucumber chips (A) and sweet pickles (B). Arrow shows acetic acid peak. First peak in both A and B is EDTA.

accordingly (external standard method). Inject 10 μL prepared sample solution and obtain amount of injected acetic acid directly from calibrated data module. Calculate acetic acid content in g/100 mL for liquid samples and in g/100 g for semisolid and solid samples. Re-inject 10 μL acetic acid–EDTA standard solution and recheck data module calibration at regular intervals.

Recovery Study

Add proper volume of acetic acid stock solution to sample so that acetic acid content of spiked sample approximately doubles. Prepare spiked sample and determine its acetic acid content as described previously. Calculate percent recovery.

Results and Discussion

The manufacturer of the LC column used in this study suggests avoiding the presence of metal ions in the system for high column efficiency (5). EDTA is a good chelating agent and was therefore added to sample solutions to a final concentration of 0.05% to mask any metal ions present. EDTA did not affect the response of acetic acid as reported previously with ascorbic acid (6), but it was essential for maintaining column efficiency and obtaining reproducible results. The EDTA peak has a shorter retention time (5.6 min) than that of acetic acid (13.9 min) and does not interfere with the quantitative determination of acetic acid. Response was linear over a range of 1–100 μg acetic acid in the presence of 0.05% EDTA. The results indicate that the method is sensitive and as little as 1 μg acetic acid (equivalent to 0.01% acetic acid content) can be determined reliably by the method.

Acetic acid was resolved as a single peak in all food products analyzed. Figures 1 and 2 show typical chromatograms. Under the conditions used in this study, the peaks of citric, ascorbic, succinic, lactic, formic, and fumaric acids were well separated from the acetic acid peak; none of these acids interfered with the quantitation of acetic acid. The identity of the acetic acid peak in the samples was confirmed by its relative retention time (relative to EDTA peak) and by spiking various samples with known amounts of standard acetic acid. The acetic acid peak had a relative retention time of 2.49 ± 0.01 in all samples analyzed, and it was the only peak increased

Table 1. Acetic acid content of vinegars

Sample	Acetic acid, g/100 mL[a]	CV, %
Rice vinegar	3.88 ± 0.18	4.6
Tarragon vinegar	4.60 ± 0.15	3.3
Wine vinegar	4.67 ± 0.12	2.6
Distilled white vinegar	4.72 ± 0.18	3.8
Apple cider vinegar	4.97 ± 0.23	4.6

[a]Average of 6 determinations ± SD.

Table 2. Acetic acid content of pickles and brines

Sample	Acetic acid content[a]	CV, %
Spanish olives	0.362 ± 0.014	3.9
Spanish olives brine	0.570 ± 0.017	3.0
Dill cucumber chips	0.494 ± 0.016	3.3
Dill cucumber chips brine	0.560 ± 0.026	4.7
Sweet pickles	1.042 ± 0.046	4.4
Sweet pickles brine	1.395 ± 0.057	4.1
Hot cauliflower	1.089 ± 0.030	2.8
Hot cauliflower brine	1.313 ± 0.045	3.5
Cocktail onions	1.455 ± 0.038	2.6
Cocktail onions brine	1.575 ± 0.043	2.8
Hot pepper rings	1.561 ± 0.038	2.5
Hot pepper rings brine	2.141 ± 0.090	4.2
Dill relish	0.452 ± 0.014	3.1
Sweet relish	0.725 ± 0.015	2.1

[a]Average of 6 determinations ± SD. Pickles, g/100 g; brines, g/100 mL.

Table 3. Acetic acid content of various products

Sample	Acetic acid, g/100g[a]	CV, %
Mayonnaise	0.225 ± 0.007	3.1
Salad dressing	0.720 ± 0.025	3.4
Ketchup	0.911 ± 0.045	4.9
Barbecue sauce	0.934 ± 0.043	4.6
Steak sauce	2.048 ± 0.050	2.4
Mustard	2.607 ± 0.082	3.2

[a]Average of 6 determinations ± SD.

Table 4. Recovery of acetic acid from various products

Sample	Recovery, %[a]	CV, %
Distilled white vinegar	100.8 ± 2.4	2.4
Spanish olives	95.5 ± 4.4	4.6
Spanish olives brine	95.4 ± 3.8	4.0
Dill cucumber chips	93.3 ± 2.3	2.5
Dill cucumber chips brine	95.5 ± 2.8	2.9
Dill relish	100.2 ± 3.2	3.2
Mayonnaise	97.6 ± 2.6	2.7
Barbecue sauce	94.5 ± 3.2	3.4
Ketchup	102.0 ± 2.4	2.4
Mustard	101.1 ± 3.4	3.4

[a]Average of 6 determinations ± SD.

in area in spiked samples. The increase in area was always proportional to the amount of standard acetic acid added.

Tables 1, 2, and 3 present results by LC for the acetic acid content of vinegars, pickles and brine, and other food products, respectively. When the acidity of vinegars (as g acetic acid/100 mL) was determined by titration with standardized 0.1N NaOH and phenolphthalein indicator, the results were always higher than were those obtained by the LC method. This indicated the presence of other titratable vinegar components that are calculated as acetic acid, and emphasized the specificity of the LC method for acetic acid.

The coefficients of variation for determination of acetic acid content ranged from 2.1 to 4.9% (Tables 1–3), which indicates that the method is precise. The recovery of acetic acid from various spiked samples (Table 4) ranged from 93.3 ± 2.3 to 102 ± 2.4%, which indicates that the method is also accurate.

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FERTILIZERS

Potassium Interferences in Flame Emission Method for Determining Sodium in Fertilizers

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Several sodium concentrations ranging from 5.2 to 20 ppm at K:Na ratios ranging from 0 to 40 were studied to determine potassium interferences in the AOAC official first action method, 2.147–2.150, for flame emission spectrophotometric (FES) determination of sodium in fertilizers. According to an analysis of variance performed with recoveries of sodium in solution, potassium interference is the same at low (5.2 ppm) or high (20 ppm) sodium concentrations but is different when the K:Na ratio is changed. A Duncan test showed that potassium interference is equivalent only in the adjacent ratios studied (0, 1, 2, 5, 10, 20, 40) and not for larger ranges. We obtained an average recovery of 98.8% using a calibration curve for a K:Na ratio from a preliminary determination with a calibration curve without potassium content. It is essential to know the potassium content of the sample to enable preparation of a calibration curve for correction. We recommend a collaborative study of NPK samples to determine whether to modify the FES method.

A comparison of 3 methods for determining sodium in fertilizers (1, 2) showed the best to be the atomic absorption spectrophotometric (AAS) and flame emission spectrophotometric (FES) methods because they were equivalent in accuracy and precision. In a collaborative comparison of these 2 methods (3), their equivalence was demonstrated, and the AAS method was adopted official first action. The study also showed that samples containing potassium gave higher results by the FES method than by the AAS method. Further study was recommended to determine the importance of the interference and to develop a procedure to eliminate it. In the present work, 2 aspects related to the problem are studied: the K:Na ratio at which potassium begins to affect sodium results, and a test of calibration curves constructed in the presence of potassium when determining sodium, so that the influence on the results due to potassium interference can be corrected by the method.

Experimental

Method

See **2.147–2.150** (4).

Apparatus

Flame photometer.—Corning, Model 400, with optical filter of 589 nm and natural gas used as fuel.

Procedure

Samples were prepared with sodium and potassium chlorides (analytical grade) of known contents within limits specified later. No NPK samples were used.

To become familiar with the method and to determine the magnitude of the potassium interference, we made some preliminary tests with solutions having K:Na ratios of 80 and 80 ppm Na. Recoveries were 130% and greater. Therefore, we decided to use sample solutions with sodium concentrations ranging from 5.2 to 20 ppm and K:Na ratios ranging from 0 to 40. We chose those values because they are the most common in NPK samples. Also, in the preliminary tests, we detected several variations in recovery for the same sodium contents. To assure that those differences were due only to potassium influence and not to the apparatus and/or the AOAC method itself, solutions with sodium concentrations and K:Na ratios specified above were prepared and each one was read 5 times. A calibration curve was generated with the average of single readings of solutions with zero ratios and was used to determine recovery for the others; Table 1 shows average recoveries and their standard deviations.

After treating the data statistically and reaching preliminary conclusions, we added a few more steps to the official method to correct sodium results, making it a requirement to know the approximate potassium content in the sample.

To test the modified procedure, we prepared 4 solutions with theoretical contents of 5.2, 10, 15.2, and 20 ppm Na and 1, 10, 20, and 40 K:Na ratios, respectively.

Samples were analyzed by the official method for sodium content (higher than theoretical) and, from the known potassium contents, K:Na ratios were calculated. New calibration curves were prepared and sample solutions were read again. Using their respective corrected calibration curves, Na contents were again calculated.

Results and Discussion

In Table 1, the standard deviation represents variations due to the apparatus and/or the method itself. The zero K:Na ratio represents the calibration curve, and it is evident that absorbance variations remain within a certain range. Recoveries at this ratio are affected primarily by this absorbance variation, which does not show any bias.

Results in Table 1 show that the higher the K:Na ratio, the greater the interference due to potassium. This is most clear in solutions at 5.2 ppm Na; those recoveries vary from 99.23 to 111.54%. Solutions with 15.2 ppm Na did not follow a regular pattern. For ratio values of unity, potassium interferences and variations, or "noise," seem to be the same; this is not true for other ratios values.

An analysis of variance (ANOVA) was carried out with the single values from Table 1 to determine whether the increase in recovery was statistically significant; Table 2 shows the results. *F*-ratio values show a significant difference between ratios, which means that recovery increases are not due only to variations observed in the first tests (0 ratios), but to potassium influence. No differences in recovery for each ratio and several sodium concentrations were found.

A Duncan test (5) was performed to determine whether all the studied ratios were different or whether some of them were equivalent; Table 3 shows results of the test.

According to those results, adjacent K:Na ratios are equivalent. This allows the use of a new calibration curve for the FES method with an approximate K:Na ratio to correct for sodium contents in samples containing potassium. An average recovery of 98.8% was obtained using a calibration curve for a K:Na ratio from a preliminary determination with a calibration curve without potassium content.

Table 4 presents sodium contents obtained by using the official method with the additional steps. It can be seen that corrected values and recoveries are in better agreement than

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Table 1. Average recoveries (%) and standard deviations (5 values) for different K:Na ratios at different sodium concentrations, using FES method

K:Na ratio		Na content, ppm 5.2	10	15.2	20
0	Av.	99.23	100.40	101.84	97.00
	SD	1.72	1.67	3.30	1.22
1	Av.	101.92	102.40	100.13	103.40
	SD	0.00	1.52	1.35	1.85
2	Av.	102.69	105.80	100.79	103.90
	SD	1.05	2.28	1.99	2.68
5	Av.	103.85	105.20	107.31	103.30
	SD	1.36	1.09	1.95	1.56
10	Av.	108.08	106.40	102.63	103.70
	SD	2.10	1.67	1.61	1.09
20	Av.	108.85	108.20	105.79	105.80
	SD	2.91	1.30	3.30	2.80
40	Av.	111.54	109.00	106.84	106.10
	SD	0.00	1.22	0.88	0.89

Table 2. Analysis of variance (5 individual recoveries) for different K:Na ratios at 4 different sodium concentrations, using FES method

Source of variance	DF	SS	MS	Variance ratio	F^a
Between concns	3	120.5	40.2	2.20	3.16
Between ratios (K:Na)	6	1082.1	180.3	9.90	2.66
Concn–ratio interaction	18	328.0	18.2	5.4	1.70
Error	112	380.7	3.4		
Total	139	1911.3			

[a]Critical values for F at 95% confidence.

Table 3. Values of Rp (least significant range between a set of means) according to Duncan test for mean recoveries at several K:Na ratios

Statistic	Ratio K:Na (ppm) 0	1	2	5	10	20	40
Recovery mean, %[a]	99.62	101.96	103.295	104.915	105.2	107.16	108.37
No. of means for a set	2	3	4	5	6	7	
Rp	2.31	2.43	2.51	2.57	2.62	2.66	

[a]Underlined recoveries are equivalent.

Table 4. Recoveries obtained for one reading by FES method, using calibration curves without and with potassium, for 4 different sodium contents

Theoretical values			Obtained values[a]			Corrected values[b]	
Na, ppm	K, ppm	K:Na ratio	Na, ppm	Na, rec., %	K:Na ratio (approx.)	Na, ppm	Na rec., %
5.2	5.2	1	5.5	105.77	1	5.0	96.95
15.2	152.0	10	16.9	111.18	9	15.1	99.34
20.0	400.0	20	21.7	108.50	18	20	100.00
10.0	400.0	40	11.6	116.00	34	10	100.00

[a]With calibration curve without potassium.
[b]With calibration curve containing potassium.

the ones obtained using a calibration curve prepared in the absence of potassium, despite the fact that K:Na ratios used to make the new calibration curves were computed inaccurately.

It is recommended that a collaborative study be done to confirm the conclusions reached by this work for NPK samples. Depending on the collaborative study results, the AOAC official first action FES method for determining sodium in fertilizers may need modification.

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Determination of Ammoniacal and Total Nitrogen in Fertilizers by Ammonia-Selective Electrode

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A method was studied for determining ammoniacal nitrogen in fertilizers by using an ammonia-selective electrode. Recovery for the method was evaluated with synthetic samples, and the method was also compared with the magnesium oxide method by analyzing 3 Magruder Check Samples. The method was adapted to determine total nitrogen in fertilizers by using the digestion procedure of the modified comprehensive nitrogen method and eliminating metal interferences by forming soluble complexes with tartaric acid. Recovery was evaluated and a comparison with AOAC official methods was made by performing a *t*-test on results for 3 Magruder Check Samples. Recoveries were good for determinations of both ammoniacal and total nitrogen by the method described, and results were not significantly different from those for AOAC methods.

In previous experimental work, Boy and Guijosa (1) studied the development of a rapid method for determining nitrogen in fertilizers by means of an ammonia-selective electrode (ASE). This electrode has been successfully used to determine ammoniacal and urea nitrogen in several products such as fertilizers (2–5), water and wastes (6), and sea water (7).

Boy and Guijosa avoided distillation in the ammoniacal nitrogen determination by making a direct measurement with the specific electrode. Performance time was improved compared with that for the AOAC magnesium oxide (MO) method (**2.065**) (8), but some results were significantly different.

Total nitrogen results were not so satisfactory, and distillation could not be avoided. The only change proposed was to substitute the final titration by an electrode measurement, with no improvement in results or performance time.

The main problem in eliminating the distillation step in the total nitrogen determination was the formation of solids when sodium hydroxide was added. This addition is necessary to increase the pH above 11, where the ammonium ion is transformed to ammonia and detected by the electrode. These solids (hydroxides of metals used as catalysts to reduce other forms of nitrogen to ammonium ions) obstructed the hydrophobic gas-permeable membrane of the electrode and results were unreliable.

We decided to continue this study, first, by testing the method for determining ammoniacal nitrogen. We then planned to study elimination of the metals used in the reduction digestion procedure, or alternatively, to find a way to keep these metals in solution above pH 11 by forming a soluble complex. This sample treatment could help eliminate distillation and permit determination of total nitrogen after catalytic digestion without metal interferences.

METHOD

Apparatus and Reagents

(a) *Ammonia electrode.*—Orion Model 95-10 or equivalent (9).

(b) *pH/mV meter.*—With expanded millivolt scale capable of 0.1 mV resolution between −200 mV and +200 mV, or specific ion meter.

(c) *Magnetic stirrer.*—Thermally insulated, with TFE-coated stirring bar.

(d) *Tartaric acid solution.*—10%. Use tartaric acid crystals to prepare aqueous solution.

(e) *Sodium hydroxide solutions.*—10N and 1N.

(f) *Ammoniacal nitrogen solutions.*—*(1)* 1000 μg N/mL. Dissolve 3.745 g reagent grade NH_4Cl in 1 L volumetric flask, dilute to volume with water, and mix. *(2)* 100 μg N/mL. Transfer 25 mL solution *1* to 250 mL volumetric flask, dilute to volume with water, and mix. *(3)* 10 μg N/mL. Transfer 25 mL solution *2* to 250 mL volumetric flask, dilute to volume with water, and mix. *(4)* 1 μg N/mL. Transfer 25 mL solution *3* to 250 mL volumetric flask, dilute to volume with water, and mix.

Preparation of Sample Solutions

Ammoniacal nitrogen.—Place sample containing 10–50 mg ammoniacal N in 250 mL volumetric flask, and add 25 mL tartaric acid solution and 100 mL water. Let stand 15 min, swirling occasionally. Dilute to volume with water, and mix.

Table 1. Samples prepared to evaluate ammoniacal nitrogen recoveries

Sample	Description	Ammoniacal N, %
1	Ammonium sulfate, reagent grade	21.20
2	Ammonium nitrate, reagent grade	17.414
3	Monoammonium phosphate, reagent grade	12.067
4	9-27-9, Synthetic sample prepd with monoammonium phosphate, sodium nitrate, potassium monobasic phosphate, and potassium nitrate, reagent grades	3.00
5	18-4-10, Synthetic sample prepd with ammonium nitrate, ammonium chloride, urea, diammonium phosphate, and potassium chloride, reagent grades	7.00

Table 2. Recovery of ammoniacal nitrogen (%) in 5 samples[a]

Sample	Av. ($n=5$)	Std dev.	Theor. value	Rec., %
1	21.290	0.107	21.20	100.424
2	17.316	0.195	17.414	99.454
3	12.21	0.055	12.067	101.185
4	3.016	0.015	3.000	100.533
5	6.914	0.038	7.000	98.771
Av. rec.				100.07
SD				0.955
$t_{calc.}$ (4 DF)				0.1639

[a]See Table 1 for sample descriptions.

Table 3. Magruder samples used to compare ASE method and MO method (ammoniacal nitrogen determination)

Sample	Description	Av. ammoniacal N, %
6	5-10-15, Magruder 7308 Check Sample	2.7040
7	28-0-0, Magruder 8308 Check Sample	8.8785
8	5-10-30, Magruder 8208 Check Sample	3.5700

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Table 4. Comparison of results for ammoniacal nitrogen (%) by ASE method and by MO method (Magruder study statistical evaluation (10))

	ASE			MO (10)				
Sample	Av.	SD	No. detns	Av.	SD	No. labs	$t_{calc.}$	DF
6	2.762	0.051	5	2.7040	0.0430	5	0.115	8
7	8.832	0.040	5	8.8785	0.5792	22	−0.100	25
8	2.996	0.040	5	3.5700	0.02828	1	−1.035	4

Table 5. Methods for determining total nitrogen in fertilizers

Method	Catalysts used	AOAC sec.
Salicylic acid (SA) method	Salicylic acid, Hg, or HgO	**2.058**
Comprehensive nitrogen (CN) method	Cr, HgO	**2.059**
Modified comprehensive nitrogen (MCN) method	Cr, $CuSO_4$	**2.061**
Raney powder (RP) method	Raney powder (50% Ni, 50% Al), HgO	**2.063**
Reduced iron (RI) method	Fe, $CuSO_4$	—[a]

[a]Not an official AOAC method.

Table 6. Samples used to evaluate total nitrogen recoveries

Sample	Description	Total N, %
9	Potassium nitrate, reagent grade	13.85
10	Ammonium nitrate, reagent grade	34.828
11	Urea, reagent grade	46.65

Total nitrogen.—Proceed as in **2.061** and **2.062** pars. 1–3 (8). Then cool flask until it can be handled without gloves, transfer contents to a 250 mL volumetric flask, cool to 25°C, dilute to volume with water, and mix. Transfer 10 mL aliquot to 100 mL beaker, add ca 60 mL water and 2.5 g tartaric acid, and adjust pH of solution to 7–8, first with 10N NaOH and then with 1N NaOH. Transfer solution to 100 mL volumetric flask, dilute to volume with water, and mix.

Determination

Connect ammonia electrode to pH/mV meter or specific ion meter, place electrode in ammoniacal nitrogen solution *4*, and warm up pH meter. Set function switch to expanded mV. Add 1.5 mL 10N NaOH to each 100 mL solution. Set reading to 000.0 by adjusting calibration. Use magnetic stirrer at constant rate throughout procedure. Samples and standards should be at same temperature. Place electrode into each solution and read mV of working and sample solutions. Plot mV reading (linear axis) against concentration (log axis) on standard 3- or 4-cycle semilogarithmic paper. Determine nitrogen content of samples from standard curve.

Results and Discussion

Ammoniacal Nitrogen

Five samples (Table 1) were prepared using reagent grade materials, and were analyzed in 5 replicates to determine method recovery. Table 2 shows percent recovery for each sample. Average percent recovery showed no significant difference at the 95% confidence level when compared with 100% by a *t*-test.

Three Magruder Check Samples (Table 3) were analyzed to compare results obtained by the ammonia-selective electrode method with those reported by the MO method in the Magruder sample statistical evaluations. Table 4 shows results obtained and *t*-test values. No significant differences were found at the 95% confidence level for samples 6, 7, and 8 for analyses by laboratories 5, 22, and 1, respectively. Results indicate that the ammonia-selective electrode method yields good recoveries and is equivalent in accuracy to the MO method.

Table 7. Recovery of total nitrogen (%) in 3 samples

Sample[a]	Av. ($n=2$)	Range	Theor. value	Rec., %
9	14.01	0.06	13.85	101.155
10	34.615	0.21	34.828	99.398
11	46.37	0.38	46.65	99.400
Av. rec.				99.984
SD				1.014
$t_{calc.}$ (2 DF)				−0.0273

[a]See Table 6 for sample descriptions.

Table 8. Magruder samples used to compare ASE method with AOAC official and reduced iron methods (total nitrogen)

Sample	Description	Approx. N, %
12	4-8-24, Magruder 8210 Check Sample	4
13	16-4-8, Magruder 8204 Check Sample	16
14	28-0-0, Magruder 8108A Check Sample	28

Table 9. Total nitrogen (%) in 3 Magruder Check Samples (ASE method)

Sample[a]	Av. ($n=3$)	SD
12	4.024	0.023
13	16.085	0.036
14	24.039	0.018

[a]See Table 8 for sample descriptions.

Total Nitrogen

Experimental work on total nitrogen determination was directed first to sample treatment after reduction digestion to find a way to eliminate the interferences caused by the metals used as catalysts. Table 5 shows 5 available methods (4 are AOAC official methods) to carry out the digestion of the sample. After a study of several complexing agents, we decided to use the digestion of the modified comprehensive nitrogen method for the following reasons: (a) Cr and $CuSO_4$ both can be masked with tartaric acid, forming soluble complexes above pH 11. (b) This method has a wide field of application. (c) It is an official AOAC method. (d) It does not have the problems associated with mercury disposal.

To evaluate the recovery of the method, 3 reagent grade samples (Table 6) were analyzed in duplicate. Table 7 shows averages, standard deviations, and percent recoveries. Average recovery showed no significant difference at the 95% confidence level when compared with 100% by a *t*-test.

Three Magruder Check Samples (Table 8) were analyzed to compare the results obtained by the ammonia-selective

Table 10. Comparison of results for ammonia-selective electrode method with those reported in Magruder statistical evaluations

Method	Statistic	Sample 12	Sample 13	Sample 14
Reduced	*t*	0.0356	−0.0410	−0.0412
ion	DF	9	8	10
Modified	*t*	−0.0589	−0.0837	−0.0726
comprehensive	DF	18	22	15
Salicylic	*t*	−0.0070	−0.0394	0.073
acid	DF	21	29	11
Devarda	*t*	0.0783	−0.152	−0.0492
	DF	10	14	3
Raney	*t*	0.206	0.155	0.251
powder	DF	3	5	7
Comprehensive	*t*	−0.0249	−0.0447	−0.306
	DF	23	23	31

electrode method with those reported in the statistical evaluation of Magruder samples. Table 9 presents the average results for these samples. Table 10 shows results of *t*-tests carried out between the ASE method and each of 6 different methods reported by Magruder participant laboratories; there were no significant differences.

In general, we can say that the ASE method for determining ammoniacal and total nitrogen in fertilizers is an adequate method that gives average results which are in good agreement with theoretical values and are also equivalent to results obtained by AOAC official methods. Important advantages of the ASE method for ammoniacal nitrogen determination are applicability to samples containing urea and a more rapid performance because of the elimination of the distillation step, which is also a major advantage when determining total nitrogen.

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WATER

Determination of Alkalinity and Acidity of Water by Conductometric Acid-Base Titration

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The alkalinity and acidity of water are parameters of great importance for studies of aquatic ecosystems. This paper describes the determination of total alkalinity by using acid-base titration with trichloroacetic acid solution as titrant. Total acidity is determined by titration with ammonium hydroxide solution. Both titrations are monitored conductometrically. Performance is evaluated and compared with performance of the potentiometric titration. Automated conductometric titration is simple, fast (30 s/titration), sensitive (detection limit 0.1 ppm $CaCO_3$), and accurate. Relative error of the determination increased from about 1% at high levels to about 10% at 1 ppm $CaCO_3$. The relative standard deviation of alkalinity measurements ranged from 1 to 5%; for acidity, these values ranged from 1.5 to 17%. Relative standard deviation reached 33% at lower detection limits. Samples containing noncarbonate contributory components of alkalinity (borate, silicate, phosphate, organic acids) can be reproducibly analyzed.

Extensive research is now being conducted to study the effect of acidic precipitation on aquatic ecosystems. Total alkalinity and acidity of the water are 2 parameters of great importance in the investigation of this problem (1). Alkalinity of water is defined as the capacity to neutralize the equivalent sum of all acids; acidity is the capacity to neutralize the equivalent sum of all bases. In other words, alkalinity is the sum of the concentrations of proton acceptors, whereas acidity is the sum of the concentrations of proton donors (2).

Classically, alkalinity and acidity in water are determined by acid-base titration with solutions of H_2SO_4 and NaOH. The equivalence points are detected by colorimetric indicator or by potentiometry with pH glass electrode. Results are reported in ppm or mg/L, expressed as $CaCO_3$ (3).

Several operational errors in the analytical determination of alkalinity and acidity are discussed in the literature (2). A major problem is definition of the titration end points. Titration using colorimetric indicators is inadequate because errors can occur in the visual detection of the end point color change. Potentiometric titration using a "total fixed end point" introduces a relative error in analysis of low alkalinity water because of uncertainty of the pH value at the end point. In water of low alkalinity or acidity, end point recognition must be very precise to obtain meaningful results.

In addition, precision and accuracy of traditional techniques are not adequate. A potentiometric titration procedure developed by Gran (4) and Larson and Henley (5) and discussed by Thomas and Lynch (6) mathematically linearizes a buffered portion of the titration curve before and after the equivalence point to characterize equivalence points. This technique improves the accuracy of the determination with respect to locating the end point. However, the basis of the technique is the actual response of the pH glass electrode which has problems of frequent and tedious calibration and slow response in the presence of other constituents of alkalinity (borate, silicate, phosphate, weak organic acids). Furthermore, the technique is associated with systematic errors; computer programs have been published to alleviate this problem (7). Several indirect methods reported to quantitate acidity have some fundamental limitations and are not recommended (8). In the present work, the acid-base conductometric titration is evaluated for determining total alkalinity (9, 10) and acidity in natural waters.

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Experimental

Reagents

All chemicals used in the measurement were analytical reagent grade. Stock solutions were analyzed by appropriate analytical methods. Synthetic sample solutions were prepared by serial dilution of stock solution. The titrant solutions 0.1 and 0.01M trichloroacetic acid (TCAA) were standardized with tris(hydroxymethyl)aminomethane and anhydrous sodium carbonate. Aqueous ammonia solutions (0.1 and 0.01M) were standardized with TCAA solution. Titrant solutions were prepared fresh daily and kept under nitrogen atmosphere. To minimize changes in sample volume, 0.01M titrant solutions were used for low levels and 0.1M solutions for high levels of alkalinity and acidity.

Apparatus

Potentiometric titrations were carried out in the conventional manner by using a Radiometer automatic titration system (Parts ABU13, PHM64, TTT60, TTA60, and REC61) and Radiometer pH glass and reference electrodes. An automatic conductometric titrator consisted of a YS132 digital conductance meter and 2 Radiometer ABU13 burets coupled to a Radiometer REC61-R1A112 recorder. All titrations were carried out at constant temperature (22 ± 0.2°C). Potentiometric and conductometric titrations were conducted simultaneously in one titration vessel.

Procedure

The electrode assembly (pH glass, reference, and conductivity cell) was immersed in 100.0 mL sample in a 150 mL beaker. The standard solution of titrant was dispensed from a 2.5 mL automatic buret, and the values of pH and relative conductivity registered automatically. It was not necessary to convert relative conductivity readings to absolute values.

To test possible CO_2 loss during the alkalinity titration, CO_2 content of a synthetic sample at pH 4 was monitored by a CO_2 gas-sensing electrode. No measurable CO_2 change occurred during the first 60 s. A 5% CO_2 loss was registered after 5 min. Because the duration of the titration is less than 60 s, there is no serious problem of distorted results.

Results and Discussion

It is well recognized that conductometric titration may be applied where potentiometric methods fail to give dependable results, for example, the direct titration of weak acids by weak bases, and the displacement titrations of salts and moderately weak acids or bases by strong acids or bases (11). These types of titrations include determinations of alkalinity and acidity. If conductivity instead of pH is followed during titration, the plot against increments of titrant is defined by straight lines whose intersection defines the end point. Only 6 points are required for such a plot. In contrast to potentiometric titration, readings near the end point have no signifi-

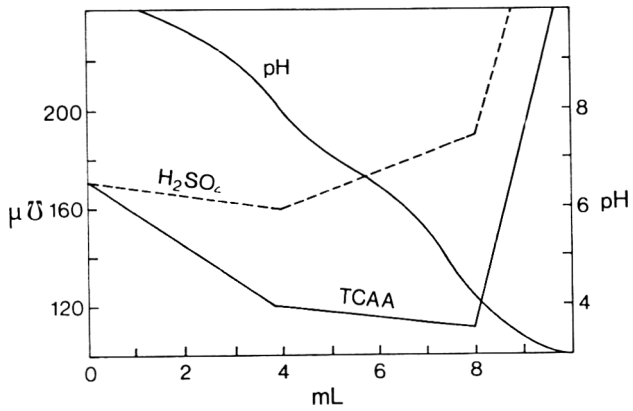

Figure 1. Potentiometric and conductometric titration curves of carbonate solution with H_2SO_4 and TCAA.

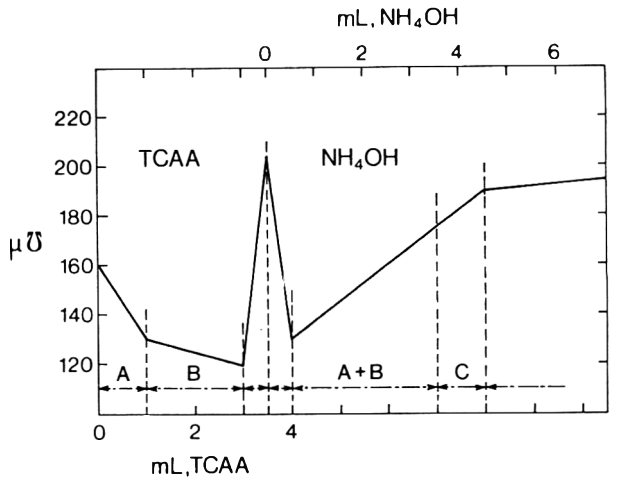

Figure 2. Schematic conductometric titration curve of alkalinity and acidity by 2 consecutive titrations.

Table 1. Alkalinity and acidity analysis of synthetic samples (ppm $CaCO_3$)

Sample	Nominal total alkalinity[a]	Nominal total acidity[b]	Mean	Rel. SD, %	Rel. error, %
1	210.0		210.5	1.1	0.25
2	105.0		104.1	1.2	0.86
3	52.5		51.0	2.9	1.0
4	21.0		20.5	2.9	2.4
5	10.5		10.4	2.9	1.0
6	5.3		5.4	3.7	1.9
7	2.1		2.0	5.0	4.8
8	1.1		1.0	5.0	10.0
9	0.5		0.55	9.1	9.1
10	0.1		0.15	33.3	50.0
11		120.0	118.0	1.5	1.7
12		60.0	60.5	1.5	0.8
13		30.0	30.8	3.2	2.7
14		12.0	11.7	4.3	2.5
15		6.0	5.8	6.9	3.3
16		3.0	3.1	6.5	3.3
17		1.2	1.2	17.0	0.0
18		0.6	0.65	15.4	8.3
19		0.3	0.35	14.3	16.7
20		0.1	0.15	33.3	50.0

[a]Alkalinity samples were Na_2CO_3 solutions containing 1% each of borate, silicate, phosphate, acetate, and citrate.
[b]Acidity samples were H_2SO_4 solutions containing 1% phosphoric acid.

cance. The effects of dissociation, hydrolysis, and solubility of the reaction products are negligible.

A major advantage of conductivity is easy location of the end point regardless of the actual pH at which it occurs. Solutions of H_2SO_4 and NaOH commonly used for alkalinity titration are not the most suitable titrants for conductometric titrations because of their high limiting equivalent conductances. Trichloroacetic acid is a strong acid (pK ≈ 0.5) and the anion has a low equivalent conductance (39.7 mho-cm²/equiv.). Similarly, ammonium hydroxide is a good candidate for acidity titration. Figure 1 illustrates the shape of conductometric/potentiometric titration curves obtained by titration of carbonate solution with H_2SO_4 and TCAA solutions.

Preliminary titrations of synthetic samples containing various constituents of alkalinity including organic compounds (beef extract, phthalate, humic and fulvic acids) showed the superiority of conductometric titration over potentiometry. Potentiometric titration produced distorted curves and indistinct end points. Response of the pH electrode was slow; a single titration required up to 30 min. Conductometric titrations gave better shaped titration curves with well defined points of inflection, and were accomplished in 1 min. Figure 2 shows the schematic conductometric titration curve of alkalinity and acidity by 2 consecutive titrations of one sample. The first equivalence point corresponds to caustic alkalinity (A); the second break indicates bicarbonate alkalinity (B). Total alkalinity is the sum of A + B volumes. The steep increase of the conductivity curve results from excess TCAA. When this is followed by titration with NH_4OH solution, the first break of the curve represents neutralization of excess TCAA. The volume of NH_4OH solution from this point to the second break point corresponds to back-titration of TCAA previously consumed by alkalinity of the sample (A + B) and to original acidity of the sample (C). This system of 2 titrations allows determination of sample acidity and alkalinity in one beaker.

To evaluate precision and detection limit of the conductometric titration, 10 replicate analyses were performed on 10 synthetic alkalinity and acidity samples. Samples were prepared from a stock solution of sodium carbonate containing 1% each of borate, silicate, phosphate, acetate, and citrate, and sulfuric acid containing 1% phosphoric acid. The initial conductivity was adjusted to 300 μS by addition of KCl solution. The results, summarized in Table 1, demonstrate a 10% relative error in the 1–10 ppm concentration range and about 1% in the 20–200 ppm range. The useful detection limit was

Table 2. Comparison of potentiometric and conductometric titrations of alkalinity[a]

Sample	$CaCO_3$ added, ppm	Mean $CaCO_3$ found,[b] ppm	RSD,[b] %	Rel. error,[b] %	Rec.,[b] %
1	1.5	1.4(1.4)	67(10)	7.3(4.0)	93(93)
2	10.0	10.4(10.2)	12.6(4.7)	4.0(2.0)	104(102)
3	12.6	12.5(12.6)	10.3(1.8)	0.8(0.0)	87(100)
4	45.0	44.0(44.3)	1.7(1.4)	3.9(3.1)	98(98)
5	150.0	149.4(148.9)	2.0(1.6)	1.3(0.9)	99(99)
6	299.0	292.6(296.6)	1.1(0.7)	2.3(1.0)	97(99)
7		1.6(1.5)	33.9(13.4)		
8		41.5(41.7)	5.8(1.2)		
9		97.3(96.9)	2.7(1.5)		
10		178.5(177.7)	1.8(1.1)		
11		204.1(203.0)	1.8(1.6)		
12		249.8(251.0)	0.8(1.5)		

[a]Results are based on 9 replicate analyses.
[b]Values in parentheses are results of conductometric titrations.

0.1 ppm $CaCO_3$. Relative standard deviations range from 15% (low levels) to 1% (high levels).

Natural water samples were selected, collected, and prepared by the Quality Assurance and Methods Section of the National Water Research Institute for the "Interlaboratory Quality Control Study" (12). The criterion for the selection was to use samples with a variety of composition, concentration, and background matrices. Six laboratories of the Water Quality Branch across Canada analyzed 8 natural samples from various regions for total alkalinity by using their standard materials.

Table 2 compares the results of the potentiometric titrations with those of conductometric titrations. In summary, conductometry is superior to potentiometry with respect to accuracy and precision. An easy and simple identification of the end point in conductometric titration contributes to its improved performance. The proposed method has simple instrumentation and operation. It covers the required concentration range and has adequate sensitivity. Even complex samples containing various contributory components of alkalinity and acidity can be reproducibly analyzed at the sampling rate of 30 s/titration.

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SUGARS AND SUGAR PRODUCTS

Gas Chromatographic Determination of Residues of Bromopropylate and Two of Its Degradation Products in Honey

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An analytical procedure is described for determination of residues of bromopropylate (BP) and its main degradation product 4,4'-dibromobenzophenone (BBP) in honey. The method also allows the determination of 4,4'-dibromobenzilic acid (BBA), a potential intermediate to BBP. The method involves dissolution of the honey in water and separation of BP and BBP from BBA on a partition column. BP and BBP are further cleaned up and separated on a silica gel column and determined by gas chromatography (GC) with electron capture (EC) detection. BBA is oxidized with potassium dichromate to BBP, which is partitioned into dichloromethane and further cleaned up on a silica gel column before determination by GC with EC detection. The method is sensitive to 0.02 mg BP or BBP/kg and 0.023 mg BBA/kg honey.

Bromopropylate, isopropyl-4,4-dibromobenzilate (BP), is the active ingredient of Folbex® VA, which was developed by Ciba-Geigy Ltd in collaboration with the Veterinary Institute, Freiburg (FRG). Folbex VA has been approved by authorities in some European countries (e.g., IKS, Interkantonale Kontrollstelle, Bern, Switzerland; BGA, Bundesgesundheitsanstalt, Berlin, FRG; etc.) as a veterinary drug for checking (diagnosis) bees for infestation with varroa mites and for control (therapy) of mite infestation in bees caused by the varroa mite (varroa disease) or the acarine mite (acarine disease) (1).

The active substance contained in the Folbex VA fumigant strip is vaporized as the strip smoulders in the hive and acts as a contact poison in the mites. The treatment is performed in the evening when all bees are inside the hives. During fumigation, the active substance was found to partially oxidize to 4,4'-dibromobenzophenone (BBP) (Figure 1). Both the active substance and BBP may contaminate the honey in the hive. As a consequence, an analytical method for the determination of both unchanged BP and BBP was required. The method also allows the determination of 4,4'-dibromobenzilic acid (BBA) as a potential intermediate to BBP, although this compound was never found in actual field samples.

METHOD

Apparatus

(a) *Rotating evaporator.*—Type Rotavap (R. Buchi, Flawil, SG, Switzerland), or equivalent.

(b) *Chromatographic glass tube.*—With stopcock, 1.4 cm id, 30 cm long.

(c) *Gas chromatograph.*—Hewlett-Packard Model 5710 A, or equivalent, equipped with ^{63}Ni electron capture (EC) detector. Operating conditions: injector 250°C, detector 300°C, argon–methane (95 + 5) carrier gas 36 mL/min. Column: borosilicate glass, 130 × 3.0 mm id, packed with 10% OV-101 on Gas-Chrom Q, particle size 0.15–0.18 mm (80–100 mesh). Temperatures: isothermal, 270°C for BBP, 280°C for BP.

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Reagents

Use analytical grade reagents throughout.

(a) *Prepacked column.*—Extrelut® (Art. No. 11737, E. Merck, Darmstadt, FRG) for elution of lipophilic compounds.

(b) *Oxidation reagent.*—2% $K_2Cr_2O_7$ dissolved in 20% aqueous sulfuric acid.

(c) *Silica gel.*—For adsorption, Woelm No. 02747, activity grade I.

(d) *Degradation product standards.*—Synthesized by Ciba-Geigy Ltd, Basel, and confirmed by elemental analysis and spectrometry.

Preparation of Samples

Take whole honey from one hive, centrifuge, and draw subsample of 500 g for analysis.

Extraction and Cleanup of BP and BBP

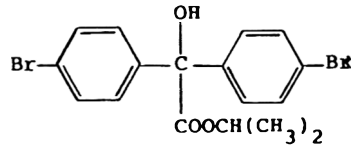
Dissolve 5 g honey in 15 mL deionized water by stirring well with glass rod, add 1 mL HCl, and pour mixture on partition column (ready-to-use Extrelut). (HCl is added for the following elution of BBA. If determination of this compound is not desired, HCl may be omitted.) Allow ca 20 min for complete penetration into adsorbent. Elute BP and BBP with four 25 mL portions of *n*-hexane and collect eluates in 100 mL beaker. Transfer contents of beaker to chromatography column, 14 mm id, previously filled with *n*-hexane and 6 g silica gel (Woelm, No. 02747, activity grade I). Let hexane percolate through column. Wash beaker and column twice with 25 mL *n*-hexane–dichloromethane (8 + 2) and discard eluate. Elute BBP with 100 mL dichloromethane into 250 mL round-bottom flask. Elute BP with 100 mL dichloromethane–acetone (8 + 3) into another 250 mL round-bottom flask. Evaporate both solvents to dryness by using rotating evaporator (water bath temperature 30–40°C). Dissolve both dry residues each in 10 mL *n*-hexane and evaporate again to dryness to eliminate traces of dichloromethane. Again dissolve both dry residues each in 10 mL *n*-hexane. Solutions are now ready for GC analysis.

Elution of BBA

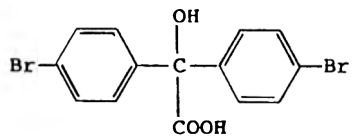
Elute remaining BBA from Extrelut column with four 25 mL portions of ethyl ether. Collect eluates in 250 mL round-bottom flask and evaporate solvent to dryness by using rotating evaporator (water bath temperature 30–40°C). Residues are not completely dry because ethyl ether carries with it some water.

Oxidation of BBA to BBP and Column Cleanup (2)

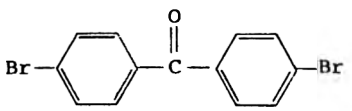
Dissolve residue in 5 mL $K_2Cr_2O_7/H_2SO_4$ reagent, let stand 10 min, and shake occasionally. Add 20 mL saturated sodium chloride solution and transfer to 100 mL separatory funnel. Rinse flask that contained residue with 25 mL dichloromethane, transfer rinse to separatory funnel, and shake. Filter organic phase through small chromatographic tube containing cotton plug and ca 20 g anhydrous sodium sulfate into 250 mL round-bottom flask. Repeat extraction two more times



BP



BBA



BBP

Figure 1. Chemical structures for bromopropylate, isopropyl-4,4'-dibromobenzilate, $C_{17}H_{16}Br_2O_3$, mol. wt 428.12 (top). 4,4'-Dibromobenzilic acid, $C_{14}H_{10}Br_2O_3$, mol. wt 386.04 (middle). 4,4'-Dibromobenzophenone, $C_{13}H_8Br_2O$, mol. wt 340.01 (bottom).

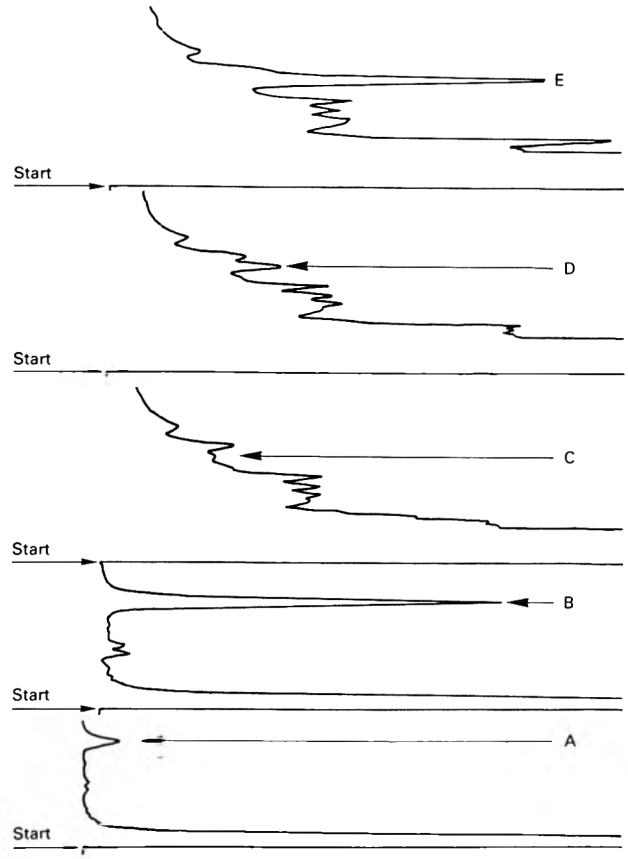


Figure 2. Typical gas chromatograms of bromopropylate: A and B, standards, 0.05 and 0.5 ng BP, respectively; C, control honey sample, aliquot injected corresponding to 2.5 mg honey; D and E, control honey samples fortified with 0.04 and 0.2 mg BP/kg, respectively, aliquot injected corresponding to 2.5 mg honey or 0.1 and 0.5 ng BP, respectively.

with fresh 25 mL portions of dichloromethane. Evaporate solvent to dryness, using rotating evaporator (water bath temperature 30–40°C). Pour 6 g silica gel (Woelm No. 02747, activity grade I) into 14 mm id chromatography column, previously filled with *n*-hexane–dichloromethane (8 + 2). Let silica gel settle and drain solvent to top of adsorbent. Dissolve residue containing BBP in 25 mL *n*-hexane–dichloromethane (8 + 2) and transfer to column. Let solvent percolate through column. Wash flask and column 3 times with 25 mL of the above solvent mixture and discard eluate. Elute BBP with 150 mL *n*-hexane–dichloromethane (6 + 4) into 250 mL round-bottom flask. Evaporate solvent to dryness, using rotating evaporator (water bath temperature 30–40°C). Dissolve dry residue in 10 mL *n*-hexane and evaporate again to dryness. Dissolve dry residue in 10 mL *n*-hexane. Solution is now ready for GC analysis.

Preparation of Calibration Curves

Determination of BBP.—Prepare 4 standard solutions containing 0.1–0.01 μg BBP/mL hexane. Inject 5 μL of each solution corresponding to 0.5–0.05 ng BBP into gas chromatograph. Measure peak heights either with a ruler or electronically and plot against ng BBP on double logarithmic scale or compute regression line. Use the same standards for determination of BBP from BBA.

Determination of BP.—Prepare 4 standard solutions containing 0.1–0.01 μg BP/mL hexane. Inject 5 μL of each solution corresponding to 0.5–0.05 ng BP into gas chromatograph. Plot ng vs peak heights as described above.

Determination of Residues in Samples

Inject 5 μL of each sample solution ready for GC analysis into gas chromatograph. Measure peak heights either with ruler or electronically and compare with corresponding standard curve either graphically or electronically.

Table 1. Typical recoveries of BP, BBP, and BBA in honey

Compd	Fort. level, mg/kg	Rec., %, SD (No. of analyses)
BP	0.04	66 ± 9.9 (n = 6)
	0.2	78 ± 3.4 (n = 7)
BBP	0.04	95 ± 5.4 (n = 6)
	0.2	92 ± 2.3 (n = 6)
BBA	0.04	70, 70
	0.2	66, 67, 69, 76
	1.0	66, 64

If sample peak is higher than highest standard peak, dilute extract with hexane and repeat injection, or inject more concentrated standard solution when working with computer.

For calculation of BBA residues, expressed in ng/mg = mg/kg: multiply ng BBP found by stoichiometric conversion factor (mol. wt BBA/mol. wt BBP = 1.1354) and divide by injected sample portion (in mg).

Control Values, Limit of Determination

Determination of BP and BBP.—Control honey samples were analyzed according to the procedure described above. When 5 μL of the final solution was injected, the chromatograms showed no significant interference at the retention times of BP or BBP. Therefore, the limit of determination may be calculated from the minimum detectable amount of BP or BBP, which is 0.05 ng for both compounds and corresponds to 0.02 mg/kg.

Determination of BBA.—When 5 μL of the final solution of control samples, analyzed as described above, was injected.

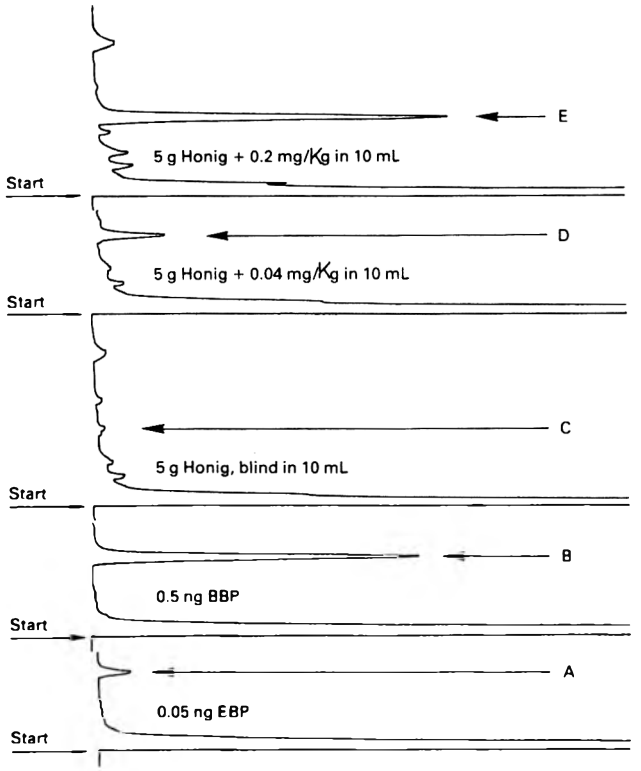


Figure 3. Typical gas chromatograms of dibromobenzophenone: A and B, standards, 0.05 and 0.5 ng BBP, respectively; C, control honey sample, aliquot injected corresponding to 2.5 mg honey; D and E, control honey samples fortified with 0.04 and 0.2 mg BBP/kg, respectively, aliquot injected corresponding to 0.1 and 0.5 ng BBP, respectively.

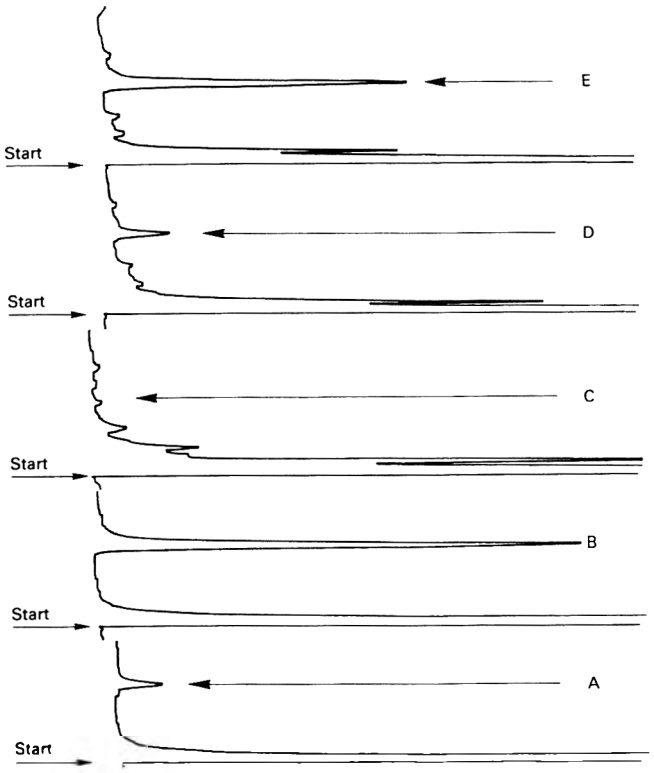


Figure 4. Typical gas chromatograms of dibromobenzophenone (BBP) originated from dibromobenzilic acid (BBA): A and B, standards, 0.05 and 0.5 ng BBP, respectively; C, control honey sample, aliquot injected corresponding to 2.5 mg honey; D and E, control honey samples fortified with 0.04 and 0.2 mg BBA/kg, respectively, aliquot injected corresponding to 2.5 mg honey or 0.1 and 0.5 ng of original BBA, corresponding to 0.088 and 0.44 ng BBP, respectively.

the chromatograms showed no significant interference at the retention time of BBP. Therefore, the limit of determination may be calculated from the minimum detectable amount of BBP, which is 0.05 ng and corresponds to 0.058 ng or 0.023 mg BBA/kg.

Results

Table 1 gives the results of recovery experiments; these data show that the developed method is suitable for the determination of bromopropylate, the degradation product BBP, and the potential intermediate BBA. The method has the advantage that the honey used for analysis is completely dissolved in water, thus avoiding the formation of a filter cake which might contain nonextractable residues. The analytical procedure described was applied with no difficulties to the routine analysis of honey samples derived from supervised trials. Small amounts of BP and BBP were found in some field samples shortly after treatment; BBA was never detected. Figures 2, 3, and 4 show some typical gas chromatograms of BP and BBP in fortified honey samples.

Acknowledgment

The skilled technical assistance of D. Schnabel is gratefully acknowledged.

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Liquid Chromatographic Determination of Lactose Purity: Collaborative Study

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Results from 8 laboratories are presented for the collaborative study of a proposed method for quantitative liquid chromatographic (LC) determination of lactose purity. Each laboratory was provided with 10 blind duplicate, edible-grade lactose samples plus a lactose reference standard and a fructose internal standard. Testing required the comparison of aqueous 2% lactose sample solution against a reference standard of equal concentration and quantitation by using a fructose internal standard. The LC system specified an isocratic acetonitrile–water (80 + 20) mobile phase, flow rate 2.0 mL/min, and chromatographic separation on a carbohydrate column. Compounds were detected by using refractive index. Statistical analysis for all study data indicates excellent between-laboratory precision with a coefficient of variation (CV) range of 0.53–1.37% and an overall average of 0.977%. Statistical analysis after excluding outliers showed equally good repeatability and reproducibility of less than 1% and 1.5% CV, respectively. The method has been adopted official first action.

Lactose is produced commercially as a by-product of the cheese industry. It is designated as fermentation grade 98%, crude grade 98.4%, edible grade 99.0%, and USP grade 99.85% (1). The producers and users of lactose have determined lactose purity by a variety of techniques (2–7); the most widely used are the AOAC reducing sugar Lane-Eynon method and the AOAC Munson-Walker chemical method (8).

When discussing these methods, many analysts express concern about the ability of available methodology to analyze lactose precisely and with sufficient sensitivity to distinguish the different grades. As a result of this concern, method development was undertaken in 1981 to provide a practical and reliable liquid chromatographic (LC) method to quantitatively determine the percent purity of lactose grades at the 98–100% level.

The developed method, after a 4-laboratory collaborative study, was presented at the 1982 AOAC international meeting (9) and recommended for official first action adoption. Although extensive validation had been performed and excellent precision data had been obtained, the method was not adopted because of insufficient number of collaborators (10). The method has been subjected to a second collaborative study, described below.

Ten collaborators were sent 10 blind duplicate, lactose edible-grade samples. The 99.0% edible-grade samples were chosen because they are most commonly encountered in our laboratory. Each collaborator was also provided with lactose reference and fructose internal standards. Each collaborator was furnished with a set of instructions to perform a system suitability check, detailed instructions for conducting the final collaborative trial, report forms, and a method of analysis.

Collaborators were instructed to analyze each of the 20 samples according to the supplied method and to quantitate the unknowns by comparison with the lactose reference standard. The study was designed under the advisement of Committee C statistician O'Donnell and Committee D statistician Ruggles in accordance with Youden and Steiner (11).

This report of the Associate Referee, J. R. Saucerman, was presented at the 97th Annual International Meeting of the AOAC, Oct. 3–6, 1983, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee D and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) 67, March issue.

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Purity of Lactose
Liquid Chromatographic Method
First Action

Principle

Lactose purity is detd by comparing aq. 2% lactose sample soln against ref. std of equal concn, using liq. chromatgy with isocratic mobile phase, carbohydrate column, and refractive index detector. Response ratio of sample lactose to internal std is compared with ratio of std lactose to internal std.

Apparatus

(a) *Liquid chromatograph.*—Hewlett Packard Model 1081, equipped with refractive index detector, autosampler, and Model 3390A integrator. Operate with column at room temp. Equiv. system may be used.

(b) *LC column.*—p-10 Carbohydrate, 4.6 mm id × 25 cm, with guard column (Whatman) or equiv. column system providing appropriate retention times and sepn for H_2O, lactose, and fructose internal std (Figure 1).

Reagents

(a) *Acetonitrile.*—LC grade (Fisher Scientific Co., or equiv.).

(b) *Water.*—LC grade.

(c) *Mobile phase (isocratic).*—CH_3CN–H_2O (80 + 20) at 2 mL/min.

(d) *Fructose internal std soln.*—Weigh 50 g fructose powd (Sigma Chemical Co. β-D(−)-fructose, or equiv.). Transfer to 500 mL vol. flask and dil. to vol. with H_2O. Prep. fresh weekly.

(e) *Lactose reference std soln.*—USP grade milk sugar free of glucose (Sigma Chemical Co., or equiv.). Accurately weigh (±0.2 mg) 2 g undried lactose std. Quant. transfer with small portions of H_2O to 100 mL vol. flask. Add 10.0 mL fructose internal std soln and dil. to vol. with H_2O. Prep. fresh daily.

System Suitability Test

(a) *Repeatability.*—Let system equilibrate with flow rate of 2 mL/min. Inject ten 25 μL aliquots of prepd std soln. Chromatogram should show baseline resolution with H_2O retention time at 1–2 min, fructose 2–3 min, and lactose 5–6 min. Coefficient of variation (CV) for peak response factors (lactose peak ht/fructose internal std peak ht) of 10 injections should be ≤0.6%. If repeatability is unsatisfactory, let system equilibrate longer, and repeat test.

(b) *Linearity of detector response.*—Perform linearity check by analyzing std lactose solns at 1.4, 1.8, 2.0, 2.2, and 2.6% concn levels. Linear regression analysis of peak ht response factor vs concn should provide correlation coefficient of 0.999. Liq. chromatograph should be checked for linearity monthly and/or when changes occur in chromatgy system (app., reagents, repairs, etc.).

Preparation of Sample

Accurately weigh (±0.2 mg) 2 g lactose sample. Quant. transfer with small portions of H_2O to 100 mL vol. flask. Add

Table 1. Data obtained in collaborative trial of LC method to determine lactose purity (duplicate samples A–J)[a]

	A		B		C		D		E		F		G		H		I		J	
Coll.	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	99.6	99.8	99.7	99.4	99.9	99.8	99.6	99.6	99.3	99.3	99.9	99.5	99.1	99.4	99.8	99.6	99.4	99.6	99.6	99.8
2	99.3	99.6	98.3	97.6	97.6	97.6	100.2	100.3	102.6	101.3	100.3	98.7	99.5	99.9	97.5	97.7	97.0	98.4	97.7	97.0
3	100.0	98.2	99.7	99.8	100.2	98.8	99.6	100.3	99.6	99.9	99.1	99.0	100.4	100.0	100.4	99.8	99.9	98.6	98.4	99.2
4	99.8	101.0	98.0	100.3	99.5	101.8	100.0	100.0	99.9	100.0	98.8	99.4	99.9	99.1	98.0	101.8	100.0	97.9	101.5	98.0
5	99.3	99.3	99.3	99.3	98.6	98.9	98.8	99.6	98.7	99.5	98.8	98.8	99.6	98.8	99.7	99.0	98.2	99.0	99.3	99.0
6	101.5	100.0	101.7	101.3	102.4	101.2	100.3	101.1	100.7	100.9	101.4	101.1	99.3	101.6	101.1	101.4	100.2	100.7	100.6	100.8
7	100.6	100.4	100.1	100.1	100.3	100.5	100.0	100.4	100.2	100.4	100.2	100.1	100.6	100.2	100.5	100.6	100.1	100.0	100.5	100.0
8	100.4	100.3	100.2	100.3	101.1	99.7	99.4	100.3	99.9	99.7	99.6	99.6	100.2	99.3	99.9	99.2	99.8	99.6	99.5	99.4
N	16		16		16		16		16		16		16		16		16		16	
Mean	99.88		99.69		99.87		99.97		100.12		99.64		99.81		99.75		99.28		99.39	
SD	0.87		1.08		1.37		0.53		0.93		0.82		0.70		1.26		1.00		1.19	
CV, %	0.87		1.08		1.37		0.53		0.93		0.82		0.70		1.26		1.01		1.20	

[a]All samples tested were 99.0–100.0% edible grade.

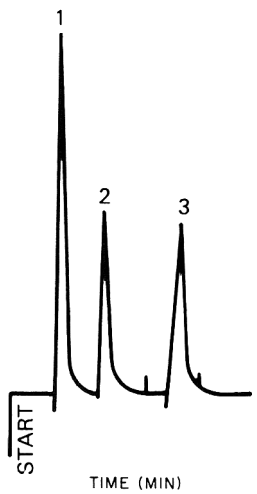

Figure 1. Typical LC peak response vs time (min) of 2% lactose solutions: 1, water 1.58; 2, fructose internal std 3.11; 3, alpha- and/or beta-lactose 5.76.

10.0 mL fructose internal std soln and dil. to vol. with H_2O. Conduct LC detn within 24 h after prepn. Note: Use same internal std soln in prepg lactose std soln and sample std soln.

Determination

Inject triplicate 25 μL aliquots of both sample and ref. std solns. If more than one sample is to be analyzed, run ref. std every third sample. Calc. results by using av. std response factors bracketing each sample set of 3.

Det. moisture content, or loss on drying for 16 h at 120° (USP, 18th rev., pp. 358, 935 (1970)), of both sample and std.

Calculation

$$\% \text{ Lactose (dry basis)} = (R/R' \times \{[W' \times (100 - M')]/[W \times (100 - M)]\} \times P$$

where R and R' = peak response factors (lactose peak to fructose peak) for sample and std solns, resp.; W and W' = wt (g) of sample and lactose std, resp.; M and M' = % moisture for sample and lactose std, resp.; P = % purity of lactose std.

Results and Discussion

Table 1 summarizes results obtained from 8 of the initial 10 collaborating laboratories. Two collaborators were unable to complete the study within the required time.

Each participant analyzed blind duplicates of samples A–J with a total of 160 analyses. All samples tested were 99.0–100.0% edible grade. The coefficients of variation for all ranged from 0.53 to 1.37% with an overall average of 0.977%.

Table 2. Statistical analysis of collaborative trial data

Sample	Lab. outliers eliminated	Estd mean rec., %[a]	Repeatability	Reproducibility
A	none	99.88	0.7378 (CV = 0.74%)	0.8767 (CV = 0.88%)
B	4[a]	99.77	0.2330 (CV = 0.23%)	1.0861 (CV = 1.09%)
C	none	99.87	0.8212 (CV = 0.82%)	1.4041 (CV = 1.41%)
D	none	99.97	0.4146 (CV = 0.41%)	0.5360 (CV = 0.54%)
E	none	100.12	0.3992 (CV = 0.40%)	0.9549 (CV = 0.95%)
F	2[b]	99.66	0.2121 (CV = 0.21%)	0.8499 (CV = 0.85%)
G	none	99.81	0.7049 (CV = 0.71%)	0.7049 (CV = 0.71%)
H	2[c], 4[b]	100.08	0.3512 (CV = 0.35%)	0.7559 (CV = 0.76%)
I	none	99.28	0.7517 (CV = 0.76%)	1.0165 (CV = 1.02%)
J	4[b]	99.34	0.3381 (CV = 0.34%)	1.1137 (CV = 1.12%)

[a]All samples were 99–100% edible grade.
[b]Cochran outlier.
[c]Dixon outlier.

Table 1 trial data were statistically analyzed, with final results shown in Table 2. The data were checked for outliers by using the Dixon, Cochran, and Youden tests. Four were eliminated as Cochran outliers and one as a Dixon outlier. The statistical analysis for recovery, repeatability, and reproducibility was performed with listed outliers omitted.

The estimated mean recovery for samples A–J ranged from 99.28 to 100.12%. Interpretation of recovery must take into consideration that all samples were edible grade with a purity claim of 99.0–100.0%. Also, samples were compared against the highest purity lactose available (USP 99.85%). Repeatability ranged from 0.21 to 0.82% CV. Reproducibility indicated a range of 0.54–1.41%.

In addition to results described in Table 2, one- and two-way analyses of variance (ANOVA) were performed. The one-way ANOVA showed $P < 0.05$ and a two-way ANOVA of $P \leq 0.0013$.

The participating laboratories used a variety of systems to perform the required tests. None reported significant difficulties. Most commented favorably on the ease and simplicity of the method. One commented on the difficulty of weighing 2.0 g ± 0.2 mg.

In conclusion, this collaborative study describes a method with excellent repeatability and reproducibility (less than 1% and 1.5%, respectively) with few outliers. These results verify the 1982 development work whereby the first collaborative study showed excellent method reproducibility ranging from 0.27 to 0.57% CV (9).

In addition to excellent precision from the 1982 and 1983 collaborative studies, the method study in 1982 defined specificity, linearity of detector response (correlation coefficient of 0.999), minimum detector response as 0.014 g/mL, working concentration range of 70–130% of the 2% sample concentration, applicability, and proof of ruggedness (9).

Recommendation

Based on the excellent precision obtained from this collaborative study and the extensive 1982 development work demonstrating specificity, precision, linearity of detector response, applicability, and ruggedness, it is recommended that this method be adopted official first action.

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PESTICIDE AND INDUSTRIAL CHEMICAL RESIDUES

Recent Advances in Cleanup of Fats by Sweep Co-Distillation. Part 2. Organophosphorus Residues

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This paper describes the isolation of 5 organophosphorus residues in beef fat by a commercial sweep co-distillation unit (Unitrex). The operating conditions specified 233°C and nitrogen flow at 230 mL/min. Recoveries of chlorpyriphos, monodechlorchlorpyriphos, bromophos-ethyl, debromobromophos-ethyl, and ethion ranged from 84 to 99%, with coefficients of variation between 3 and 5%.

Residues of organochlorine and organophosphorus pesticides are detected in Australian meat fat, the latter being derived almost exclusively from the need to dip or spray cattle in some areas of Northern Australia to control tick *(Boophilus microplus)* infestation. Accurate, reliable, rapid, and relatively inexpensive analytical methods are required to efficiently analyze large numbers of samples, particularly those destined for export. In conjunction with capillary column gas chromatography, sweep co-distillation has proven very effective in this multi-residue program over 9 months' operation.

METHOD

Reagents and Apparatus

(a) *Sweep co-distillation apparatus.*—Unitrex (Universal Trace Residue Extractor) supplied by Scientific Glass Engineering (SGE) Pty, Ltd, Australia. This unit has been described previously (1).

(b) *Florisil.*—60–100 mesh (Floridin Co., Pittsburgh, PA) 1.0% deactivated as prepared in ref. 1.

(c) *Sodium sulfate.*—Granular, anhydrous, AR grade. Soxhlet-extract with dichloromethane for 2 h, air-dry, and heat at 130°C for 2 h.

(d) *Trap preparation.*—Pack sodium sulfate/Florisil separately, as for organochlorine residues, but in the ratio 1 + 1. Use short strand variety of glass wool for plugs in each end of trap, and pack so that it completely fills B5 cone area. Failure to do this results in air bubbles being trapped in cone area during solvent elution of trap, with subsequent lengthy elution times.

(e) *Solvents.*—Hexane, dichloromethane (Mallinckrodt Nanograde), and ethyl ether (redistilled, peroxide-free).

(f) *Gas chromatography.*—Varian 3700 fitted with ^{63}Ni electron capture detector, 12 m × 0.33 mm id vitreous silica column BP 10 (Scientific Glass Engineering Pty, Ltd). Operating conditions as described in ref. 1.

Preparation of Fortified Meat Fat

Beef fat (200 g), previously analyzed to confirm that no organophosphorus residue above 0.05 mg/kg was present, was fortified with 2.0 mL mixed pesticide standard in hexane to produce a fat containing chlorpyriphos (*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate, 0.16 mg/kg), bromophos-ethyl (*O*-(4-bromo-2,5-dichlorophenyl) *O,O*-diethylphosphorothioate, 0.32 mg/kg), ethion (*O,O,O',O'*-tetraethyl *S,S'*-methylene bisphosphorodithioate, 0.24 mg/kg), debromobromophos-ethyl (*O*-(2,5-dichlorophenyl) *O,O*-diethylphosphorothioate, 0.47 mg/kg), and monodechlorchlorpyriphos (*O,O*-diethyl-*O*-(3,6-dichloro-2-pyridyl) phosphorothioate, 0.41 mg/kg). The fat was then mixed in a beaker 20 min at 60°C, using a magnetic stirrer/hot plate.

Procedure

The analysis procedure was exactly as previously reported (1), except that the detachable trap was packed with sodium sulfate and Florisil in the ratio 1 + 1. See discussion for further details.

Results and Discussion

The 5 organophosphorus pesticides used in this investigation are those most frequently detected in meat fats in this laboratory in the past 10 years. Debromobromophos-ethyl (DBB) and monodechlorchlorpyriphos (DCC) apparently result from dehalogenation of their respective parent compounds, bromophos-ethyl and chlorpyriphos (2, 3). These 2 dehalogenated residues are now quantitatively reported in routine samples.

Table 1 shows details of the recovery data. All compounds are recovered in yields above 84%. In experiments with chlorpyriphos, bromophos-ethyl, and ethion, recoveries were dependent on the amount of sodium sulfate in the traps (see Table 2). By changing the ratio of sodium sulfate/Florisil from 1 + 3, as for organochlorine residues, to 1 + 1, we observed consistently higher chlorpyriphos recoveries. Elimination of sodium sulfate resulted in complete loss of chlorpyriphos and ethion, and significant reduction in bromophos-ethyl recovery. By packing the traps with an intimate mixture of sodium sulfate and Florisil (1 + 1), rather than separate portions of the 2 materials, almost complete loss of chlorpyriphos and bromophos-ethyl occurred.

By decreasing the amount of Florisil below the 50% level, the trap eluate contained co-extracts which adversely affected the solvent front area of the gas chromatograms. In routine work, we pack the traps with sodium sulfate and Florisil (1 + 3), as recommended previously for organochlorine residues, and, if chlorpyriphos is detected, we repeat the analysis with the modified trap composition (1 + 1).

The explanation for the results shown in Table 2 is not fully understood at this time, but clearly the ratio of sodium sulfate and Florisil, and the activity of the Florisil, are critical in obtaining reliable recovery.

Figure 1 shows a typical chromatogram of an extract from a meat fat spiked with chlorpyriphos, bromophos-ethyl, and ethion. Eluting immediately after bromophos-ethyl is *p,p'*-DDE, which is present as a background residue in the blank fat sample. Figure 2 shows the chromatogram from a meat fat sample fortified with debromobromophos-ethyl and monodechlorchlorpyriphos. Figure 3 is a chromatogram of a typical

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Table 1. Recoveries of organophosphorus residues in spiked meat (beef) fat, using Unitrex

Pesticide	Spike level, mg/kg	Mean rec., %	SD	CV, %
Chlorpyriphos[a]	0.16	83.5	4.4	5.3
Bromophos-ethyl[a]	0.32	96.6	2.9	3.0
Ethion[a]	0.24	99.0	5.2	5.2
DBB[b]	0.47	95.6	3.5	3.7
DCC[b]	0.41	91.9	3.6	3.9

[a]Results based on 27 recoveries.
[b]Results based on 10 recoveries.

Table 2. Variation in recoveries (%) of organophosphorus residues with different ratios of sodium sulfate and Florisil[a]

Sodium sulfate–Florisil[b]	Chlorpyriphos	Bromophos-ethyl	Ethion
Florisil only	not recovered	77	not recovered
1 + 9	60	94	86
1 + 2	58	89	81
1 + 1	79	97	100
2 + 1	86	98	103
1 + 1[c]	<10	81	<5

[a]Unitrex conditions: 230°C, nitrogen flow 230 mL/min, single analysis.
[b]Sodium sulfate and 1.0% deactivated Florisil in separate portions, unless otherwise specified.
[c]Sodium sulfate and Florisil intimately mixed before packing in traps.

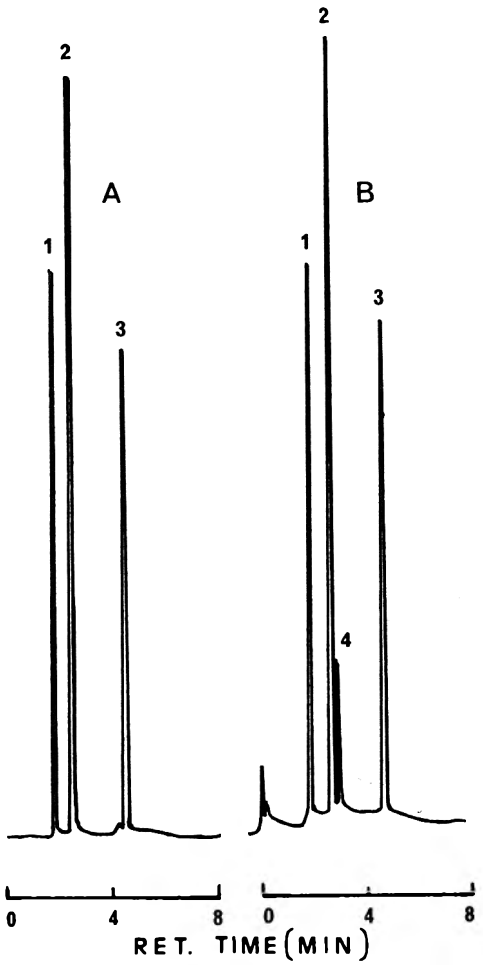

Figure 1. Gas chromatograms of standards and cleaned up extract from spiked meat fat: A, Organophosphorus pesticide standards: (1) chlorpyriphos (0.15 ng), (2) bromophos-ethyl (0.2 ng), and (3) ethion (0.3 ng). B, Recovery extract: (1) chlorpyriphos, (2) bromophos-ethyl, (3) ethion, and (4) *p,p'*-DDE as background residue in blank meat fat.

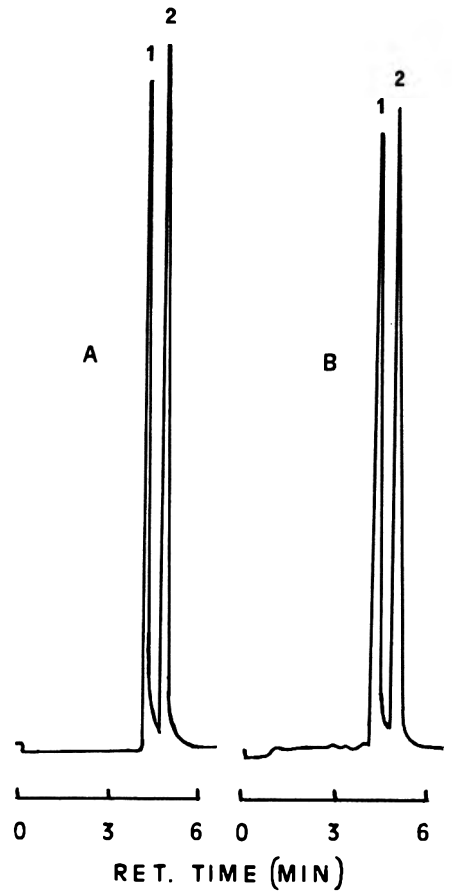

Figure 2. Gas chromatograms of standards and cleaned up extract from spiked meat fat: A, Organophosphorus pesticide standards: (1) DBB (0.47 ng), and (2) DCC (0.41 ng). B, Spiked meat fat extracts: (1) DBB, and (2) DCC.

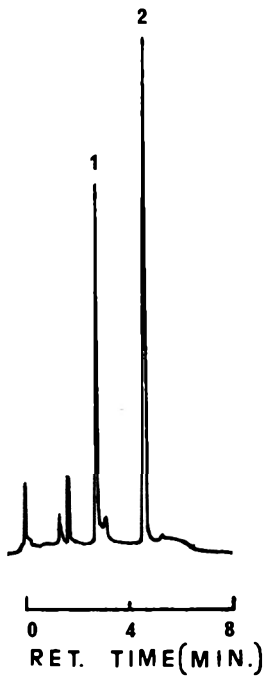

Figure 3. Gas chromatogram of typical sample extract containing (1) *p,p'*-DDE, and (2) ethion residues.

meat fat sample containing 0.21 mg/kg of ethion, and 0.04 mg/kg of *p,p'*-DDE.

So far in our monitoring program, we have used one fused silica column (12 m × 0.33 mm id) containing a medium polarity bonded phase (BP 10), and an electron capture detector. The fact that over 2000 samples have been injected on this system, without any major contamination problems, is an indication of the cleanliness of the Unitrex extracts. Introduction of a sample split system at the capillary column outlet would permit more specific, simultaneous detection of organochlorine and organophosphorus pesticides, respectively, with an electron capture and N/P detector.

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Gas Chromatographic and Mass Spectrometric Determination of Ametryn and Its *N*-Dealkylated Products

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Gas chromatographic and mass spectrometric properties of ametryn and its *N*-dealkylated products were studied to establish the potential use in a recently reported method describing the residue analyses of these compounds by gas chromatography with N-P detection. Electron impact mass spectra show base peaks at the molecular ion (M^+), and methane chemical ionization mass spectra give base peaks at (M + 1) ion for all the compounds studied. Characteristic mass spectral fragmentations of ametryn, GS-11354, GS-11355, and GS-26831 are presented. Combined gas chromatography–mass spectrometry rather than gas chromatography alone provides unambiguous residue characterization. The technique also allows quantitation of ametryn and its *N*-dealkylated products that cochromatograph with interfering materials present in a complex substrate.

Ametryn (2-methylthio-4-ethylamino-6-isopropylamino-*s*-triazine) is an economically important herbicide used for effective control of most annual broadleaf and grassy weeds in a wide variety of crops. Such *s*-triazine herbicides accounted for 28% of herbicide manufacture (1) in 1974.

Ametryn can be photodegraded, biodegraded, or metabolized to a number of *N*-dealkylated products. For regulatory purposes in the United States, determination of ametryn residues in different substrates must be augmented by the additional determination of the 3 known metabolites, viz., 2-methylthio-4-amino-6-isopropylamino-*s*-triazine (GS-11354), 2-methylthio-4-amino-6-ethylamino-*s*-triazine (GS-11355), and 2-methylthio-4,6-diamino-*s*-triazine (GS-26831) (Ciba-Geigy Corp., 1980, personal communication), because of their potential mammalian toxicity as carcinogens. Although residue analysis for the parent compound is widely known (2, 3), an analytical method measuring its metabolites is lacking in the literature. A previous communication (4, 5) from this laboratory described a facile gas chromatographic (GC) method for residue analysis of these chemicals in tropical root crops, taniers, yams, and cassava. In the present work, we report the mass spectral properties of ametryn and its metabolites under electron impact (EI) and methane chemical ionization (CI) mass spectrometry, and propose GC and mass spectrometry (MS) for complete and unequivocal characterization and determination of the herbicide and its degraded products.

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METHOD

Reagents

Pesticide-grade solvents were used throughout.

Standards.—Ametryn was obtained from U.S. Environmental Protection Agency; GS-11354, GS-11355, and GS-26831 were gifts from Ciba-Geigy Corp., PO Box 11422, Greensboro, NC 27409.

Standard solutions.—Stock solutions.—12 mg ametryn/25 mL ethyl acetate–toluene (3 + 1 v/v); 9 mg GS-11354/25 mL ethyl alcohol; 5.9 mg GS-11355/25 mL ethyl alcohol; 4.4 mg GS-26831/25 mL ethyl alcohol. *Working solutions.*—Make appropriate dilutions as needed in the same solvent.

Gas Chromatography

A Hewlett-Packard Model 5840A gas chromatograph equipped with nitrogen-phosphorus detector Model 18847A and a glass column, 70 cm × 2 mm id, packed with 5% DEGS-PS on 100–120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA 16823) was used. Parameters were as follows: gas flows (mL/min): helium carrier 30, hydrogen 3, air 50; temperatures (°C): column oven 200, injector 200, detector 300. Voltage to the detector was adjusted for a current of 1.6×10^{-11} amp or 10% AUFS at attenuation 2^5.

Mass Spectrometry

A Finnigan 4021 GC/MS instrument in the positive ion mode was used. Samples were introduced through the gas chromatograph equipped with a 183 cm × 2 mm id glass column of 3% OV-17, under the following conditions: helium carrier gas flow 20 mL/min, injection volume 1 μL, injection port temperature 200°C, and column oven temperature programmed from 150 to 225°C at 10°/min. Electron impact mass spectra were obtained at 70 eV. Chemical ionization mass spectra were obtained by using methane reagent gas at ionizing gas pressure 0.2 torr.

Results and Discussion

Ametryn and the 3 known degraded products GS-11354, GS-11355, and GS-26831 are analyzed by GC with nitrogen-phosphorus detection and a column of 5% DEGS-PS; Figure 1 represents a typical chromatogram. As low as 0.3 ng each compound gives an easily quantitatable, discrete peak. The detection limit, expressed as ppm or mg/kg in different sub-

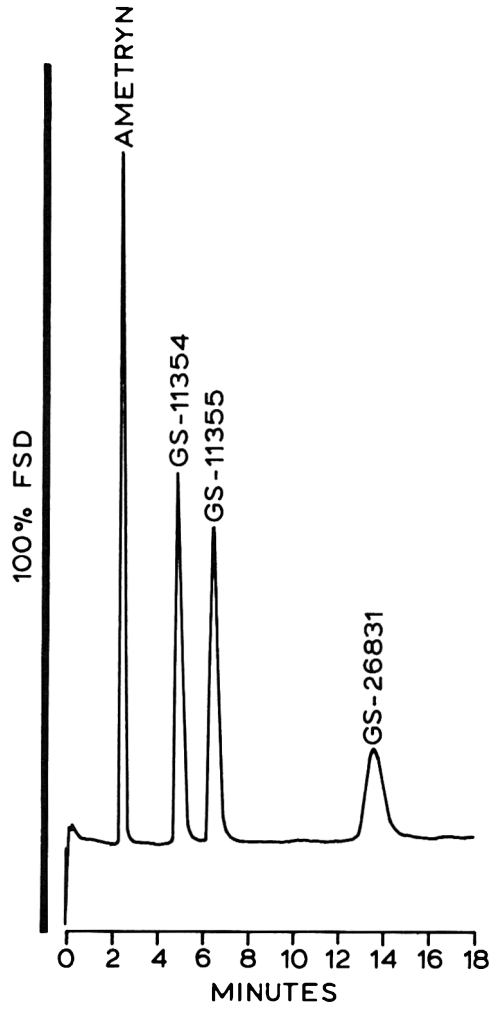

Figure 1. Gas chromatogram of mixture of standards (2 ng each); attenuation 2^5, column packing 5% DEGS-PS. For details see *Method*

strates, depends on a number of factors, viz., efficiency of the solvent system used for extraction, effectiveness of the cleanup procedure, interferences contained in the cleaned up extract, GC injection volume, and amount of substrate represented in the aliquot. A GC injection volume representing 10 mg substrate could give a detection limit of 0.03 ppm or 0.03 mg/kg. A previous communication from this laboratory reported (4, 5) a detection limit of 0.0024 mg/kg for ametryn and its metabolites in tropical root crops, taniers, yams, and cassava, by using ethyl acetate–toluene (3 + 1 v/v) extraction, cleanup by gel permeation chromatography on Bio-Beads S-X3 resin, and subsequent GC analysis of an injection aliquot representing 125 mg crop. Recoveries from fortified crops were reported (4, 5) to range from 67 to 111% at levels of 0.1–1 mg/kg.

Figures 2–5 represent the EI mass spectra of ametryn, GS-11354, GS-11355, and GS-26831, respectively, while Figures 6–9 represent the methane CI mass spectra of the same compounds.

The EI-induced fragmentations of ametryn are similar to its chlorine-containing analogs and other disubstituted *s*-triazines reported (6) in the literature. The molecular ion ($M^{\cdot}$) at m/z 227 is also the base peak. It further decomposes with loss of CH_3 or CH_3—$CH=CH_2$ to give the ions m/z 212 and m/z 185, respectively, while the loss of both gives the m/z 170 ion. The McLafferty rearrangement ion at m/z 185 expels SCH_3 to give the m/z 138 ion. A slightly different fragmentation pattern was noted (7) by other workers who used different instruments and operating parameters. The methane CI mass spectrum of ametryn gives an $(M+1)^+$ ion at m/z 228, which is also the base peak, and also gives the $(M+C_2H_5)^+$ ion at m/z 256 and $(M + C_3H_5)^+$ ion at m/z 268 to confirm 227 as the molecular weight.

The EI mass spectrum of GS-11355 gives the molecular ion and base peak at m/z 185. Loss of CH_3 and $CH_2=CH_2$ gives the m/z 170 and m/z 157 ions, respectively, and loss of both

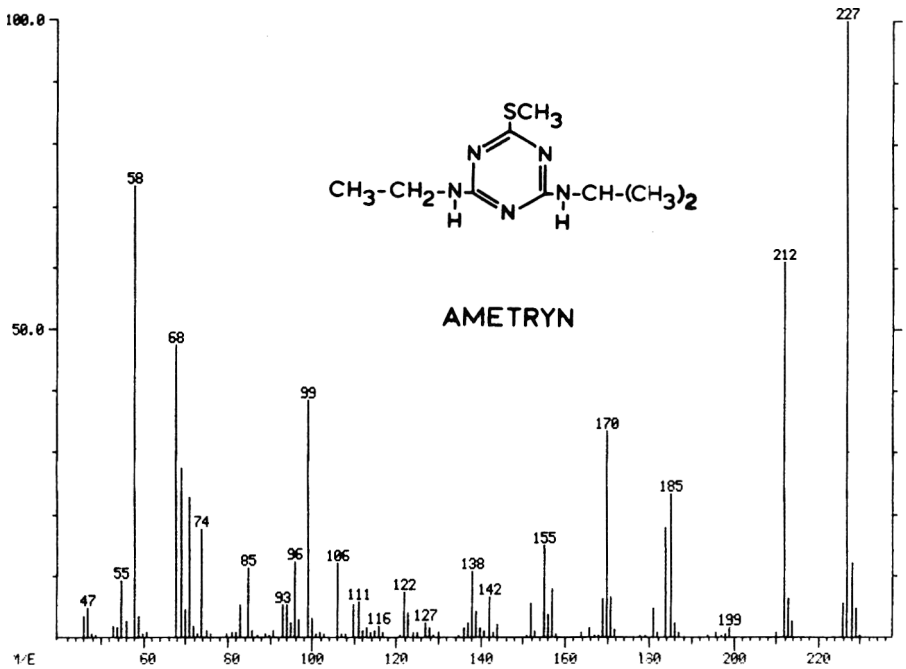

Figure 2. Electron impact mass spectrum of ametryn, recorded from 45 to 300 mass units.

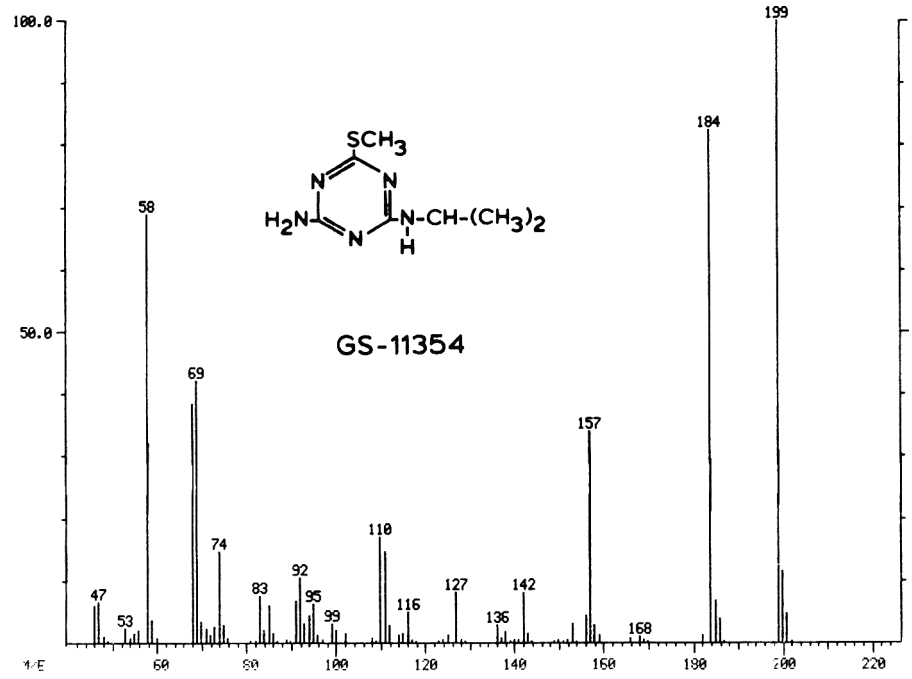

Figure 3. Electron impact mass spectrum of GS-11354, recorded from 45 to 250 mass units.

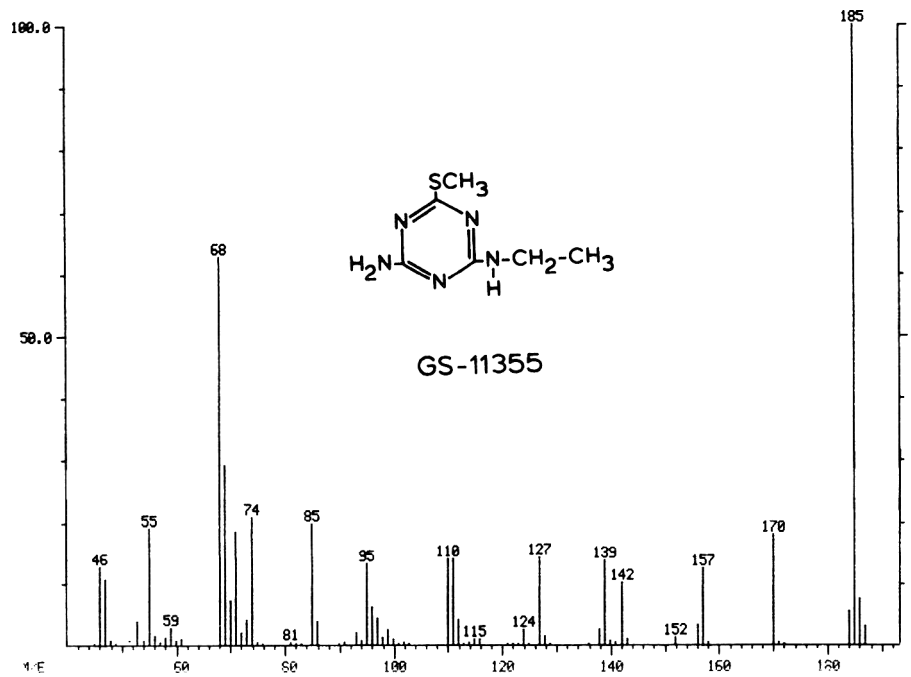

Figure 4. Electron impact mass spectrum of GS-11355, recorded from 45 to 200 mass units.

entities produces the m/z 142 ion. The ion at m/z 110 can be explained on the basis of expulsion of SCH_3 from the m/z 157 ion. The methane CI mass spectrum of GS-11355 (molecular weight 185) conforms to the typical pattern with $(M+1)^+$ ion at m/z 186 and the sequence $(M+29)^+$ and $(M+41)^+$ ions.

The EI fragmentations of GS-11354 (molecular weight 199) are also similar to those of the above compounds. The molecular ion is also the base peak. With a loss of CH_3, the ion m/z 184 appears. The molecular ion also loses CH_3—CH = CH_2 to generate the m/z 157 ion which further expels SCH_3 to give the m/z 110 ion. The methane CI mass spectrum gives the typical pattern with the base peak and $(M+1)^+$ ion at m/z 200, and the sequence $(M+29)^+$ at m/z 228 and $(M+41)^+$ at m/z 240.

The EI mass spectrum of GS-26831 (molecular weight 157) exhibits the base peak and molecular ion. However, the fragmentation pattern influenced by the absence of *N*-alkylated chains furnishes spectral lines unlike those of its counterparts mentioned earlier. The ion at m/z 111 can be rationalized by the expulsion of SCH_3 and simultaneous addition of a proton

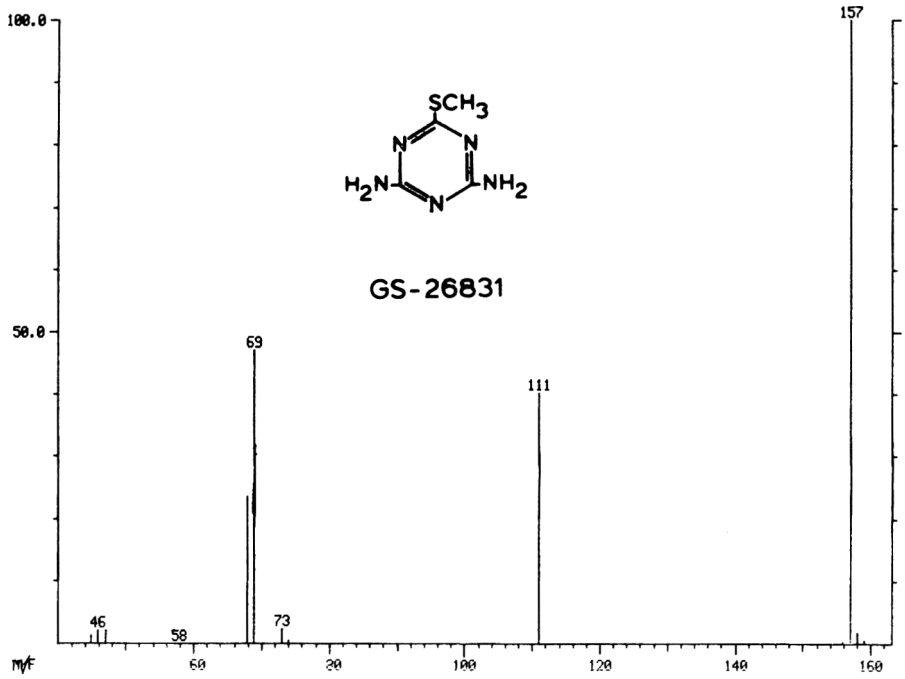

Figure 5. Electron impact mass spectrum of GS-26831, recorded from 45 to 200 mass units.

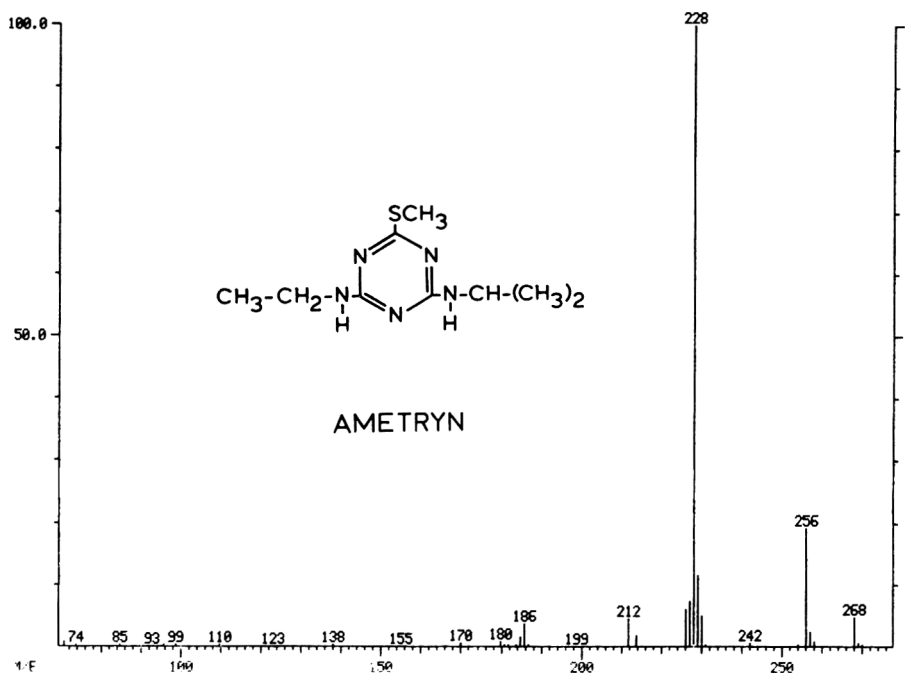

Figure 6. Chemical ionization mass spectrum of ametryn with methane reagent gas, recorded from 70 to 300 mass units.

in the molecular ion. The methane CI mass spectrum exhibits a typical sequential pattern that confirms the molecular weight of GS-26831.

It is worthwhile to mention that, in the absence of exact mass measurements and/or linked scans for confirmation, the assignments of various fragmentation ions are only tentative. The methane CI mass spectra of the chemicals studied are dominated by their pseudomolecular ions, $(M + I)^+$. By selective monitoring of this ion in GC/MS, the detection limit was 0.5 ng for the chemicals studied. This limit corresponded to 0.005 mg/kg when 100 mg substrates were used as the injection aliquot. Plotting reconstructed ion current with specified injected quantities against mass spectral scan numbers and retention times, with peaks identified by scan numbers, gave reproducible results.

The mass spectral sensitivity of ametryn and its *N*-dealkylated products in the positive ion mode of electron impact and/or chemical ionization mass spectrometry has potential

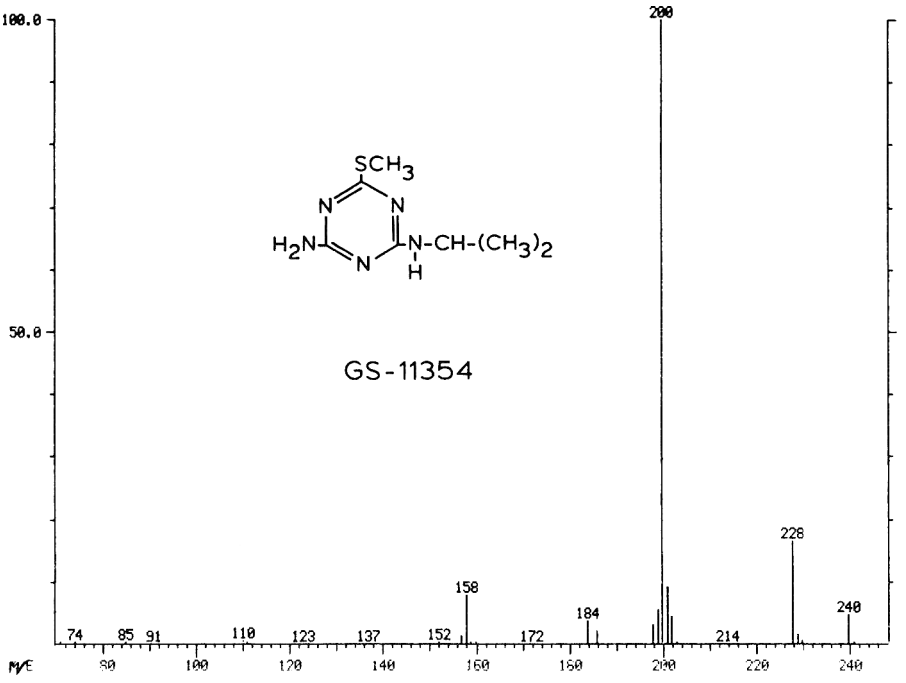

Figure 7. Chemical ionization mass spectrum of GS-11354 with methane reagent gas, recorded from 70 to 250 mass units.

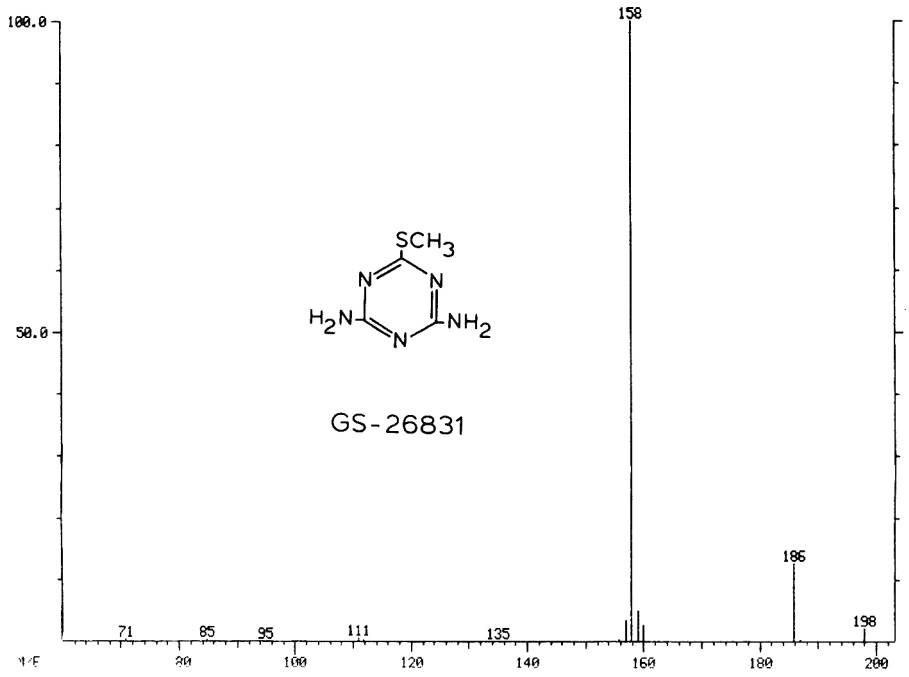

Figure 8. Chemical ionization mass spectrum of GS-11355 with methane reagent gas, recorded from 70 to 250 mass units.

advantageous use for detection of the herbicide and its metabolities by selective ion monitoring in low concentration and in complex matrices However, for precise confirmation, spectra must be obtained in the presence of co-extractives, a task precluded in the present work due to absence of any such interferences in the substrates studied, viz., taniers, yams, and cassava.

Acknowledgments

The authors thank Ciba-Geigy Corp. for the dealkylated ametryn standards. Part of this research forms the method development work in connection with IR-4 project Nos. 473, 489, and 490.

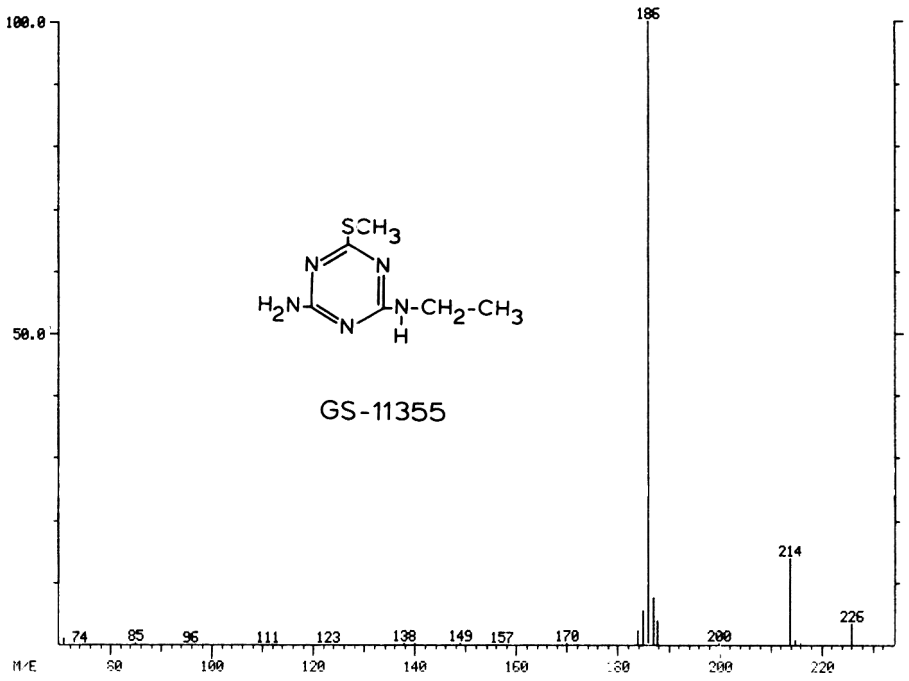

Figure 9. Chemical ionization mass spectrum of GS-26831 with methane reagent gas, recorded from 70 to 200 mass units.

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Determination of Captan, Folpet, and Captafol in Fruits and Vegetables, Using Two Multiresidue Methods

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Two multiresidue methods, the Mills method and the Luke et al. method, are widely used for the determination of pesticides in foods. These methods were evaluated for the determination of the fungicides captan, folpet, and captafol in selected fruits and vegetables. The analytical behavior of standards through these methods was investigated first. Recoveries from apples, strawberries, lettuce, and tomatoes fortified with these compounds at levels of 0.2–5.9 ppm were then obtained. The analytes were quantitated by gas chromatography with electron capture detection, using a column of 5% SP-2401 on 100–120 mesh Supelcoport. Recoveries of captan, folpet, and captafol from fortified crops ranged from 69 to 78, 90 to 93, and 67 to 83%, respectively, by the Mills procedure and from 87 to 102, 81 to 106, and 91 to 109%. respectively, by the Luke et al. method modified to include additional solvent elution of the optional Florisil column.

Captan (*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide), folpet (*N*-(trichloromethylthio)phthalimide), and captafol (*N*-(1,1,2,2-tetrachloroethylthio)-4-cyclohexene-1,2-dicarboximide) are used as broad-spectrum protective fungicides for the control of various diseases of agricultural crops. The 3 compounds are structurally related, having in common the phthalimide-type skeleton. Gas chromatographic–electron capture detection (GC-ECD) methods have been described for determining the individual fungicides (1–6). Simultaneous determination by GC-ECD of captan, folpet, and captafol in various crops has been described by Pomerantz et al. (7) and Baker and Flaherty (8). Liquid chromatographic separation of the 3 fungicides by using photoconductivity detection (9) and photoconductivity in tandem with ultraviolet detection (10) has also been reported.

We recently reported the GC behavior of the 3 fungicides on stationary phases of various polarity (11). The column we had found to be most suitable for the quantitation of these compounds was used for the GC-ECD determinations made in the present investigation.

The official multiresidue method for organochlorine and organophosphorus pesticides (12), often referred to as the Mills method, and the method of Luke et al. (13, 14) are routinely used for residue surveillance by the Food and Drug

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Administration (FDA). These methods have been adopted in Vol. I of the FDA *Pesticide Analytical Manual* (PAM I) (15). The Mills method is primarily useful in the determination of relatively nonpolar chemicals, whereas the method of Luke et al. is potentially capable of determining almost all nonpolar as well as the most polar pesticides. Minimal data are available concerning the recoveries of captan, folpet, and captafol through the 2 procedures. The purpose of the present work was to determine the suitability of these methods for the quantitative determination of the 3 fungicides in selected fruits and vegetables.

The crops selected for this study represented 2 categories each of fruits and vegetables: large, firm fruits (apples), small, soft fruits (strawberries), leafy vegetables (lettuce), and nonleafy vegetables (tomatoes). One or more of the 3 fungicides is commonly applied to all of these crops (16). The current Environmental Protection Agency (EPA) tolerances range from 0.25 ppm captafol in apples to 100 ppm captan in lettuce. Tolerances are generally in the range of 15–50 ppm for most fruits and vegetables. However, in the authors' experience, residues of these compounds are generally less than 5 ppm and often less than 1 ppm. Fortifications were therefore made at levels more consistent with these expected findings. Accurate quantitation of residues detected at much higher levels would likely require adjustment of the sample size and/or extraction solvent volume to yield a concentration in the ppm range evaluated here.

Experimental

Reagents and Apparatus

(a) *Stock solutions.*—Individual solutions of each of the 3 fungicides (100 μg/mL benzene) prepared from EPA reference standards and stored in refrigerator at −7°C.

(b) *Fortifying solutions.*—Mixtures of the 3 compounds prepared by diluting suitable aliquot of each stock solution with isooctane for use with the Mills method, or with acetone for use with the Luke et al. method.

(c) *Standard solution for GC quantitation.*—Mixture containing ca 0.2 μg/mL each of captan and folpet and ca 0.4 μg captafol/mL prepared from stock solutions by dilution with isooctane.

(d) *Gas chromatograph.*—Hewlett Packard 5880A equipped with ^{63}Ni constant current electron capture detector and Level 4 data station. Carrier gas argon–methane (95 + 5) at flow rate of 30 mL/min. Temperatures (°C)—injector 200, detector 340, oven 175 for captan and folpet and 195 for captafol. Output of detector adjusted to produce ca 40 and 60% full scale recorder deflections at oven temperatures of 175 and 195°C, respectively, for 0.5 ng chlorpyrifos.

(e) *GC column.*—Glass, 6 ft × 2 mm id, packed with 5% SP-2401 on 100–120 mesh Supelcoport. Column was prepared and conditioned as previously described (11).

Procedure

Both methods were evaluated following the procedures as described in PAM I (15). Florisil column cleanup procedures used with the Mills method (sec. 212.1) included both the specified 3-stage ethyl ether–petroleum ether elution systems (sec. 211.14d), and the alternative 3-stage methylene chloride–hexane–acetonitrile elution system (sec. 252.101). The optional Florisil column cleanup (sec. 212.24) specified in the Luke et al. method (sec. 212.2) was modified to include elution with 200 mL 50% ethyl ether in petroleum ether in addition to the specified single elution with 200 mL 15% ethyl ether in petroleum ether. The Florisil used was fully activated and tested for suitability as specified in the methods.

Each method was evaluated by first determining the analytical behavior of added standards, in the absence of any crop material, through the aqueous-organic partitioning step and the Florisil column cleanup step individually. Subsequently, recoveries from fortified samples of blended, homogenized composites of fresh produce were determined by each method. Fortifications were made directly onto the crops before the blending operation, and the fortified samples were taken through the entire method including extraction, aqueous-organic partitioning, and Florisil column cleanup. All determinations were made in duplicate.

Results and Discussion

Mills Method

A single partitioning of added standards from the aqueous acetonitrile phase (200 mL acetonitrile and 80 mL water with no crop present) with 100 mL petroleum ether, as specified by the method, resulted in average recoveries of 35.6% (35.5, 35.7), 77.4% (76.4, 78.4), and 53.3% (53.9, 52.7) of added captan, folpet, and captafol, respectively (Standards were added at levels of 20–40 μg, equivalent to 0.2–0.4 ppm in 100 g sample.) Four additional petroleum ether partitionings (100 mL each) extracted a total of 73.8% (72.7, 75.0) captan, 98.0% (96.3, 99.6) folpet, and 84.4% (83.9, 84.8) captafol. Since captan and captafol are more polar than folpet, considerable amounts of the former 2 compounds were retained in the aqueous acetonitrile phase even after 5 extractions with petroleum ether.

Elution characteristics and recovery of standards from the Florisil column, using both ethyl ether–petroleum ether elution and the alternative elution system, are summarized in Table 1. Recoveries of captan and captafol were complete in the 50% ethyl ether eluate but were significantly lower in the No. 3 alternative eluate. Folpet recoveries were complete in both systems but were split between the second and third eluates in each case.

Recoveries from fortified crop samples (100 g each), using a single 100 mL petroleum ether extraction of the aqueous acetonitrile phase as specified by the Mills method, are shown in Table 2. Recoveries of captan and captafol were erratic and generally incomplete using either of the 2 Florisil elution systems. Folpet recoveries exceeded 90% with the ethyl ether–petroleum ether system but were considerably lower with the alternative system. Significant losses, especially for captan and captafol, were anticipated in view of the low recoveries obtained in the aqueous acetonitrile–petroleum ether partitioning study on standards alone, as discussed earlier. The recoveries of all 3 compounds through the entire Mills method increased substantially in the presence of crop substrate compared with the recoveries of standards alone through the aqueous acetonitrile–petroleum ether partitioning step. The extractability of these relatively polar compounds by the Mills method is apparently affected to significantly different degrees by the presence of different crop matrices. When used for the determination of nonpolar pesticides, the Mills method consistently yielded complete (~ 90% or better) recoveries from fortified fruits and vegetables in the authors' laboratory.

Luke et al. Method

The latest revision of the Luke et al. method (14), which is described in sec. 232.4 of PAM I (15), permits rapid residue screening without Florisil cleanup in many cases. This is

Table 1. Recoveries (%)[a] of added standards from Florisil column, using (A) ethyl ether–petroleum ether (Mills method) and (B) alternative methylene chloride–hexane–acetonitrile elution systems

		Eluate				Eluate			
		6%	15%	50%	Total	1[c]	2[d]	3[e]	Total
Fungicide	Added, μg[b]	Elution System A				Elution System B			
Captan	20	0, 0	0, 0	104, 101	104, 101	0, 0	0, 0	81.1, 74.6	81.1, 74.6
Folpet	20	0, 0	63.5, 47.9	25.3, 52.5	88.8, 100.4	0, 0	88.4, 84.3	7.4, 7.9	96.2, 92.2
Captafol	40	0, 0	0, 0	97.6, 91.2	97.6, 91.2	0, 0	0, 0	83.8, 86.9	83.3, 86.9

[a]Duplicate determinations.
[b]Standards were added in 1.0 mL isooctane to Florisil column that was prewashed with petroleum ether for system A and with hexane for system B.
[c]20% methylene chloride in hexane.
[d]50% methylene chloride–0.35% acetonitrile–49.65% hexane.
[e]50% methylene chloride—1.5% acetonitrile–48.5% hexane.

Table 2. Recoveries (%)[a] from fortified crop samples analyzed by Mills method, using both (A) specified ethyl ether–petroleum ether and (B) alternative methylene chloride–hexane–acetonitrile elution systems for Florisil column cleanup

Fungicide	Crop	Added, ppm	System A	(Eluate)[b]	System B	(Eluate)[c]
Captan	apples	0.20	78.5, 69.0	(50%)	45.0, 49.5	(3)
	lettuce	0.20	ND	—	50.2, 50.5	(3)
	strawberries	0.49	74.4, 72.8	(50%)	ND	—
Folpet	apples	0.20	80.4, 67.1	(15%)	73.9, 82.0	(2)
			13.1, 22.8	(50%)	7.5, 4.3	(3)
			93.5, 89.9	Total	81.4, 86.3	Total
	lettuce	0.20	ND		80.9, 70.8	(2)
					5.0, 3.0	(3)
					85.9, 73.8	Total
	strawberries	0.49	76.3, 73.4	(15%)	ND	
			15.0, 16.8	(50%)		
			91.3, 90.2	Total		
Captafol	apples	0.24	80.7, 83.1	(50%)	85.4, 89.6	(3)
	tomatoes	0.40	71.3, 67.0	(50%)	79.8, 69.5	(3)

[a]Duplicate determinations.
[b]Captan and captafol eluted in the 50% eluate only. Folpet eluted in the 15 and 50% eluates only.
[c]Captan and captafol eluted in eluate 3 only. Folpet eluted in eluates 2 and 3 only.

particularly advantageous for compounds not recoverable from Florisil or charcoal columns, but requires the use of a more specific GC detector. The absence of Florisil cleanup often results in matrix effects on quantitation and in rapid column deterioration. Captan, folpet, and captafol were completely recoverable through Florisil cleanup, which permitted the use of ECD and eliminated any apparent matrix effects on column or detector performance. Inclusion of the optional Florisil cleanup in this evaluation was therefore deemed desirable.

Extraction of added standards from the aqueous acetone phase (200 mL acetone and 80 mL water with no crop present) with petroleum ether and methylene chloride, as specified by the method, resulted in recoveries ranging from 98.1 to 101.1% for captan, 100.1–103.6% for folpet, and 103.5–106.4% for captafol. (Standards were added at levels of 20–590 μg, equivalent to 0.2–5.9 ppm in 100 g sample.)

Recoveries of added standards (in the absence of crop material) through the combined aqueous to organic partitioning step and optional Florisil cleanup step are shown in Table 3. Although the Florisil cleanup in the Luke et al. method specifies a 15% ethyl ether in petroleum ether elution only, a 50% ethyl ether elution was added since it was found to be required for complete recoveries of the fungicides from the Florisil column. The elution patterns differ between the 2 methods because the Luke et al. method specifies placing the extract on the Florisil column in a 10% acetone in petroleum ether solution, whereas the Mills method specifies a petroleum ether solution at that stage. Due to the presence of the relatively polar acetone, the fungicides elute faster in the Luke et al. method. The result is a splitting of captan and captafol between the 15 and 50% ethyl ether eluates, and a larger portion of folpet in the 15% eluate as compared with the Mills method.

The data in Table 3 also suggest that the fungicides elute faster as a result of increasing their amounts on the column, i.e., increases of more than 20-fold in added amounts resulted in 5–15% increases in recoveries in the 15% ethyl ether eluates. This may be related to the adsorptive capacity of the Florisil column.

Recoveries from fortified crop samples (100 g each) are shown in Table 4. The apples and strawberries used in this study each contained about 0.04 ppm of field-incurred residues of captan, and recoveries were therefore corrected accordingly. With the exception of the slightly lower recoveries of captan and folpet from apples, all recoveries exceeded 90%. A 25-fold increase in the fortification level (from 0.2 to > 5 ppm in tomatoes) caused no significant difference in recoveries. The presence of crop substrate had no apparent effect on the Florisil column elution patterns relative to standards alone, nor were the individual fungicide recoveries through the entire method seriously affected by crop material.

Summary and Conclusions

Due to their relatively high polarity, captan, folpet, and captafol were only partially extracted (< 80%) in the aqueous acetonitrile to petroleum ether partitioning step specified in the Mills method. Recoveries of these compounds were therefore incomplete through the Mills method, but were significantly enhanced when co-extracted plant material was

Table 3. Recoveries (%)[a] of added standards through Luke et al. method, using modified Florisil column elution system[b]

Fungicide	Added, μg	Eluate 15%	Eluate 50%	Total
Captan	20	53.4, 52.9	41.8, 41.2	95.2, 94.1
	536	62.1, 54.3	35.2, 35.0	97.3, 89.3
Folpet	20	82.8, 84.6	17.0, 14.7	99.8, 99.3
	420	101.2, 94.9	0, 0	101.2, 94.9
Captafol	37	41.1, 41.9	56.2, 53.4	97.3, 95.3
	588	56.6, 50.1	44.1, 43.9	100.7, 94.0

[a]Duplicate determinations.
[b]Modified to include 200 mL 50% ethyl ether–petroleum ether eluate in addition to specified 15% eluate.

Table 4. Recoveries from fortified crop samples analyzed by Luke et al. method, us ng modified Florisil column elution system

Fungicide	Crop	Added, ppm	Recd, %[a]
Captan	tomatoes	0.20	101.1, 100.5
	tomatoes	5.36	102.5, 98.9
	apples	0.20	86.6, 91.0
	lettuce	0.20	92.0, 92.0
	strawberries	0.40	91.0, 90.3
Folpet	tomatoes	0.20	103.0, 105.6
	tomatoes	4.20	104.3, 101.4
	apples	0.20	81.4, 84.9
	lettuce	0.20	90.4, 97.0
	strawberries	0.40	98.7, 101.3
Captafol	tomatoes	0.37	101.6, 98.6
	tomatoes	5.88	108.9, 104.6
	apples	0.37	96.8, 93.3
	lettuce	0.37	91.2, 90.6
	strawberries	0.75	92.2, 97.5

[a]Duplicate determinations. Results presented are summed totals of recoveries in 15% ethy and 50% ethyl ether in petroleum ether.

present, i.e., in fortified crop samples. All 3 compounds were completely recovered (> 90%) through the Mills method Florisil column cleanup step using the specified 3-phase ethyl ether–petroleum ether elution system, but substantial losses of captan and captafol occurred with the use of the alternative methylene chloride–hexane–acetonitrile elution system.

The Luke et al. method, which specifies the use of the more polar methylene chloride extraction solvent for the aqueous to organic partitioning step, provided complete recovery (~ 100%) of all 3 compounds. However, substantial recovery losses occurred, especially for captan and captafol, with the use of the optional Florisil column cleanup step specified in the Luke et al. method. This was surmounted by the inclusion of a final Florisil column elution with 200 mL 50% ethyl ether in petroleum ether (i.e., the final eluate in the Mills solvent system) in addition to the 15% ethyl ether elution specified by Luke et al. The combination of the Luke et al. method, additional elution of the optional Luke et al. Florisil column with the Mills 50% ethyl ether eluate, and GC-ECD determination using an SP-2401 packing (as previously described by the authors (11)) provides reliable quantitative determination of captan, folpet, and captafol in extracts of a variety of fruits and vegetables. A major advantage attained from using multiresidue methods for this analysis is that it permits the concurrent, routine screening for numerous other pesticides of interest, provided their analytical behavior through the methods has been established.

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COSMETICS

Liquid Chromatographic Determination of Benzoyl Peroxide in Acne Preparations

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A rapid, precise, and accurate liquid chromatographic (LC) method is described for the determination of benzoyl peroxide (BP) in acne preparations. BP is extracted from a water dispersion of the preparation with dichloromethane (DCM), and an aliquot is eluted from a C-18 reverse phase LC column with acetonitrile–0.10M aqueous $NaClO_4$. Selective and sensitive quantitation is accomplished with a reductive mode electrochemical detector. This detector is an order of magnitude more sensitive than a 240 nm UV absorption detector; the lower limit of detection is 2 ng for a 4 μL injection. The recovery of BP is 99.4% and the detector response is linear to at least 2 μg per 4 μL injection.

Benzoyl peroxide (BP) is used in acne creams and lotions because it acts as an irritant to the skin, producing high epithelial cell growth rates and promoting sloughing and expulsion of comedones (1). Conventional methods for the assay of BP in such preparations include spectrophotometry using *N,N'*-di(2-naphthyl)-phenylene-1,4-diamine reagent (2), iodometric titration (3), and polarography (4).

Liquid chromatography (LC) with a UV detector has been applied to the determination of BP in acne preparations (5, 6). The use of LC with an electrochemical (EC) detector, however, offers selectivity and sensitivity improved over the published methods.

METHOD

Apparatus and Reagents

(a) *Liquid chromatograph.*—Laboratory-assembled from Varian Model 8500 pump; Rheodyne Model 7125 injection valve; Micromeritics Model 786 UV detector set at 240 nm; and custom-built (of Delran polyacetal) 3-electrode EC detector similar in design to the Bioanalytical Systems' EC detector, with ⅛ in. diameter (0.08 sq. cm) Au amalgam working electrode, 1 mm diameter Pt auxiliary electrode, and Ag/AgCl (3.5M KCl) reference electrode. Working electrode potential maintained at −0.15 V vs reference with Princeton Applied Research Model 364 polarographic analyzer potentiostat in normal dc mode. Voltammetric reduction currents measured with Keithley Instruments Model 150B amplifier. UV detector placed downstream of EC detector. Separate Linear Instruments Model 1200 strip-chart recorders used for each detector.

(b) *LC column.*—Whatman Partisil 10/25 ODS-3, 25 cm × 4.1 mm id. Operating conditions: flow rate 90 mL/h; temperature ambient; chart speed 4 min/cm. Retention time of BP ca 15 min.

(c) *LC mobile phase.*—Distilled-in-glass acetonitrile (Burdick and Jackson Labs, Inc., Muskegon, MI 49442)–0.10M $NaClO_4$ (50 + 50) purged in pump reservoir with high purity N_2 for 30 min before use to remove dissolved O_2.

(d) *Working standard solution.*—BP purified by precipitation from $CHCl_3$ solution by addition of methanol. *Caution:* Dry BP may explode spontaneously (7). Suspend 50 mg BP (accurately weighed) in 50 mL water. Add 50.0 mL dichloromethane (DCM) (Burdick and Jackson Distilled-in-Glass) and shake to extract BP. Withdraw lower DCM layer with syringe and store in 50 mL volumetric flask.

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Extraction

Accurately weigh samples of lotion or cream containing ca 5 mg BP into 16 × 150 mm screw-cap culture tubes. Add 5 mL water and shake to dissolve or disperse. Add 5 mL DCM and shake 10 min on automatic shaker. Centrifuge 3 min at 2000 rpm to separate layers. Withdraw lower DCM phase with syringe and transfer to clean 16 × 150 mm culture tubes. Store capped at 5°C until ready for injection into LC apparatus.

Liquid Chromatography

Inject exactly 4 μL aliquots of standard solution into LC. Measure BP peak heights to ± 0.5 mm and calculate average peak height, h_s. Similarly, inject exactly 4 μL aliquots of each product extract solution and determine average peak height, h_p. Calculate % BP = $w_s(h_p/h_s)(1/w_p) \times 100$, where w_s is wt (mg) of pure BP extracted into 50.0 mL DCM used to prepare standard, and w_p is product sample wt (mg).

Iodometric Titration

For comparison with an independent method (3), each product was assayed titrimetrically in quadruplicate as follows: Accurately weigh sample of acne cream or lotion equivalent to ca 250 mg BP into 100 mL beaker. Add 30 mL acetone and stir to dissolve. Transfer mixture quantitatively to 100 mL volumetric flask, rinsing beaker with four 10 mL portions of acetone, and dilute with acetone. Pipet 10.0 mL acetone solution into 125 mL Erlenmeyer flask, add 2 mL 20% KI solution, and let stand 15 min in dark. Add 25 mL acetone and titrate liberated iodine with standard 0.01N $Na_2S_2O_3$ to disappearance of yellow I_2 color. Repeat procedure on standard solution containing 250 mg pure BP in acetone.

Linear Range

Prepare 4 BP solutions containing 550, 110, 22, and 4.4 ng/μL by dissolving 5.5 mg BP in 10 mL DCM and making 3 successive 1:5 dilutions with DCM. Inject 4 μL aliquots into LC system, repeating each 5 times, and determine average peak heights.

Potential Interferences

Dissolve 8 mg BP, 5 mg benzoic acid, 6 mg perbenzoic acid, 7 mg benzoic acid anhydride, 6 mg benzil, and 80 mg benzene in 5 mL DCM. Inject 2 μL aliquot into LC system.

Partition Study

Dissolve 50 mg (accurately weighed) BP in 50.0 mL DCM. Inject exactly 4 μL into LC system. Inject exactly 4 μL working standard solution into LC system. Repeat both injections 3 times and determine average peaks.

Recovery Study

Suspend 2 g (accurately weighed) lotion in 100 mL water with shaking, and extract with 100 mL DCM, proceeding as above. Suspend 1 g (accurately weighed) lotion to which has been added 50 mg (accurately weighed) BP, in 100 mL water, and proceed as above. Inject into LC system exactly 4 μL aliquots of these DCM solutions and BP standard solution.

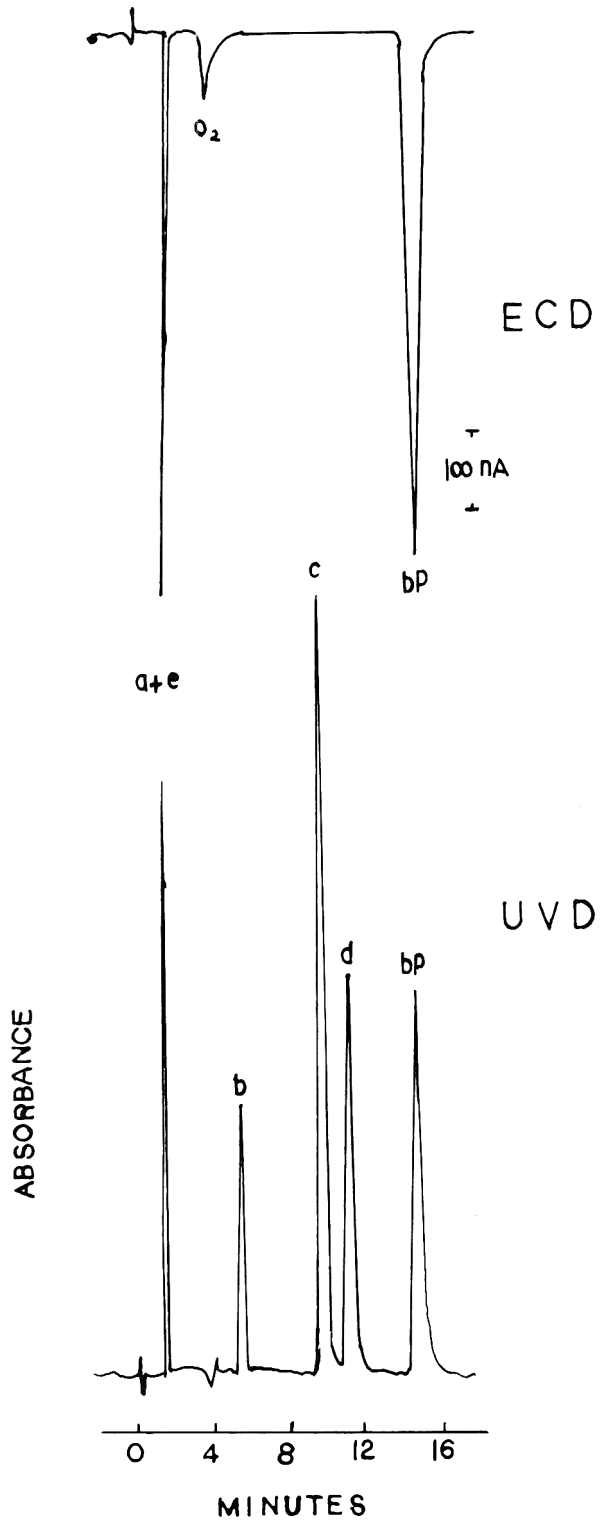

Figure 1. Separation of potential interferences by using ECD (upper) and UVD (lower). ECD sensitivity, 2 μamp full scale; UVD sensitivity, 0.50 AUFS. Peak identification: (a) benzoic acid; (b) benzene; (c) benzil; (d) benzoic acid anhydride; (e) perbenzoic acid; and (bp) benzoyl peroxide.

Repeat injections 6 times, average peak heights, and calculate percent recovery.

Results and Discussion

Selectivity of the LC system and specificity of the EC detector relative to the UV detector are evident in Figure 1. The UV-absorbing compounds shown are conceivable impurities or decomposition products of BP. All are well separated from the BP peak. The EC detector responds only to BP, perbenzoic acid, oxygen dissolved in the sample, and, with much reduced sensitivity, to benzoic acid, at −0.15 V vs the reference electrode. Specificity could be increased by decreasing the applied potential at the expense of sensitivity to BP; however, the LC separation obviates the need for this.

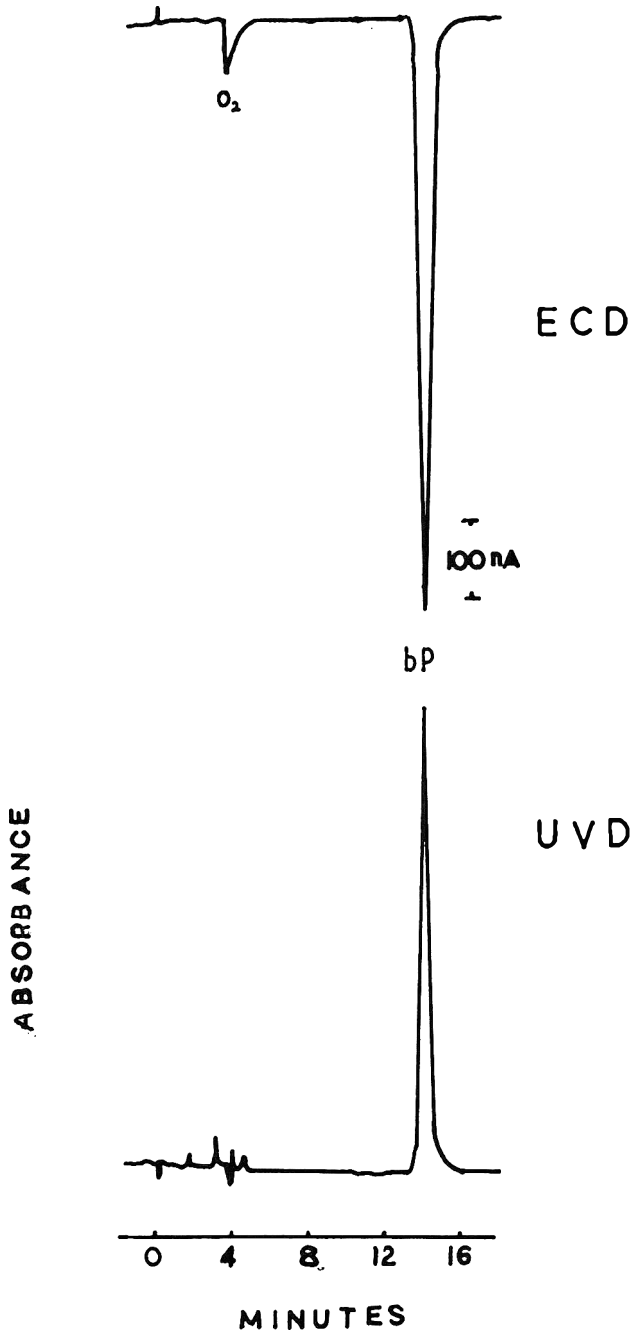

Figure 2. Chromatogram of BP in DCM extract of product 2: ECD (upper) and UVD (lower). ECD sensitivity, 1 μamp full scale; UVD sensitivity, 0.50 AUFS.

Figure 2 shows chromatograms of product 2 extracts measured on both detectors. No differences in peak area from a particular detector were noted if the sequence of detectors in the flow stream was reversed, indicating insignificant alteration of BP in either detector. In practice, either detector could be used alone unless one needed the additional sensitivity and specificity of the EC detector.

The plot of peak height vs weight BP injected is a straight line for both detectors up to at least 2 μg BP injected. Detection limits, expressed as weight BP producing a peak height

Table 1. Analysis of 4 acne preparations for benzoyl peroxide (BF)

	% BP (std dev.)[a]		
Product	LC-ECD	LC-UVD	Iodometry
1	11.3 (0.301)	11.3 (0.288)	11.2 (0.0753)
2	11.6 (0.232)	11.9 (0.313)	11.3 (0.0696)
3	5.05 (0.203)	5.08 (0.262)	5.03 (0.0300)
4	5.17 (0.185)	5.13 (0.214)	5.10 (0.0254)

[a]Based on 5 injections each of triplicate samples of each product, and 4 iodometric titrations each.

twice the noise level, are 2 ng per 4 μL injection for the EC detector and 20 ng per 4 μL injection for the UV detector.

The partition study showed 98.5% transfer of BP from aqueous to DCM phases, i.e., essentially quantitative extraction. The recovery of BP from the spiked sample was 99.4%.

Table 1 gives the results of analysis of 4 acne products by the LC method with each detector and by iodometric titration. The first 2 products contain nominally 10% BP and the other 2 contain nominally 5% BP, by label. The results of the 3 methods are in agreement for each product. There is no statistical difference in the precision of the 2 LC methods.

Acknowledgment

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OILS AND FATS

Liquid Chromatographic Determination of Adulteration of Sesame Oil

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A liquid chromatographic (LC) procedure is presented for quantitative determination of adulteration of sesame oil with soybean oil. A portion of the oil is dissolved in chloroform, diluted in the eluant 2-propanol–acetone–methanol–acetonitrile (1 + 2 + 3 + 4), and subjected to reverse phase LC. A linear calibration curve is prepared by chromatographing known mixtures of the 2 oils and plotting the volume percent of sesame oil against the peak height ratio of a selected pair of peaks. The relative standard deviation, based on 4 determinations of each of 8 sample mixtures, is less than 2.5% and the correlation coefficient is 99%. Because the LC curve for a given vegetable oil is characteristic and reproducible, the procedure can be extended to detect the adulteration of sesame oil with oils other than, or in addition to, soybean oil. An example of adulteration with rapeseed oil, in addition to soybean oil, is discussed.

Sesame seed oil is widely used for cooking oriental foods and in salad dressing formulations. The popularity of oriental foods in the United States is increasing, and there has been an increasing demand for sesame oil, resulting in importation of greater amounts. As has been observed for olive oil, demand and high price can lead to the adulteration of imported oil by mixing with more plentiful, less expensive oils.

U.S. Customs Service laboratories must be aware of adulteration of vegetable oils, because an artificial mixture of oils is dutiable at a rate not less than that for the component subject to the highest rate. Thus, the presence of soybean oil in a mixture with sesame oil will result in an increase in duty from 0.7¢ per pound (pure sesame oil) to 22.5% of the value of the shipment, a very significant increase.

For many years, naturally occurring fats and oils have been characterized and identified by their fatty acid composition, using gas chromatography (1, 2). Recently, liquid chromatography (LC), in the reverse phase mode, has been used for rapid separation of the natural triglycerides by acyl chain length and degree of unsaturation (3–8).

This paper describes a relatively simple and rapid method for the qualitative detection of adulteration of sesame oil and the quantitative determination of the extent of adulteration when the adulterant is soybean oil.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Waters Associates Model 201, or equivalent, equipped with Model 6000A solvent delivery system (pump), Model U6K universal injector, and Model R401 differential refractive index detector (Waters Associates, Milford, MA 01757). Operate chromatograph isocratically at flow rate of 1 mL/min, ambient temperature.

(b) *Chromatographic column.*—Supelcosil LC-8 column, 15 cm × 4.6 mm (id), or equivalent (Supelco Inc., Supelco Park, Bellefonte, PA 16823).

(c) *Recorder.*—Omniscribe dual pen recorder or equivalent, set at 0.5 cm/min (Houston Instrument, One Houston Sq, Austin, TX 78753).

(d) *Ultrasonic cleaner.*—Model SS-116 (Sonix IV, Inglewood, CA 94303). For degassing solvent.

Reagents

(a) *Solvents.*—Distilled-in-glass 2-propanol, acetone, methanol, and acetonitrile (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442); chloroform, hexane, reagent grade (Mallinckrodt Inc., St. Louis, MO 63147).

(b) *LC eluant.*—2-Propanol–acetone–methanol–acetonitrile (1 + 2 + 3 + 4, by volume), premixed, allowed to stand overnight, and degassed before use.

(c) *Oil extracts (standards).*—Crush a quantity of commercial dry soybeans or unroasted sesame seeds in large, metal mortar. Extract ground seeds 6 h with hexane in Soxhlet extractor. Filter extract into beaker, dry on steam bath until odor of hexane has disappeared, and transfer oil to screw-cap vial.

Preparation of Calibration Curve

(a) *Stock solution.*—Pipet 0.2 mL of each extracted (standard) oil into clean, dry test tube and dilute with 0.2 mL $CHCl_3$. Prepare fresh for each curve.

(b) *Working solution.*—Using syringe, pipet appropriate amount (μL) of stock solution of each oil into clean, dry test tube to prepare binary mixtures containing 20–70% sesame oil by volume, at 10% increments. Dilute with 100 μL $CHCl_3$ and 800 μL LC eluant and mix thoroughly by shaking. Concentration of oil in working solution is comparable to that used for sample analysis (see *Analysis.*) Prepare fresh working solution for each curve.

(c) *Calibration curve.*—Inject 20 μL each working solution into LC apparatus. Chromatogram shows peaks of separated triglycerides present and is similar to those shown in Figure 1 (sesame oil) and Figure 2 (soybean oil). Ratio of peak 2 (C_{42}) height to peak 4 (C_{46}) height is linear when plotted against volume percent of sesame oil.

Analysis

Pipet ca 50 μL oil into clean, dry test tube. Dissolve oil in 200 μL $CHCl_3$ and dilute with 800 μL LC eluant. Inject 20 μL sample (solution) into LC system. Calculate observed peak height ratio (C_{42}/C_{46}) and determine volume percent sesame oil present from calibration curve.

Results and Discussion

Figures 1 and 2 show the distinctly different chromatograms of pure sesame and soybean oils. Under the chromatographic conditions used here, it has been established that the saturated triglycerides are separated according to the total number of carbons present in the acyl chains. The presence of an *unsaturated* fatty acid in the triglyceride, however, will contribute to the total number of carbons as though the acyl chain were composed of 2 fewer carbons for each double bond present (3–9). The assignment of an equivalent acyl carbon content, or "ECN" (5), to the chromatographic peaks observed for sesame seed oil and soybean oil was confirmed in this study by the chromatographic behavior of the known

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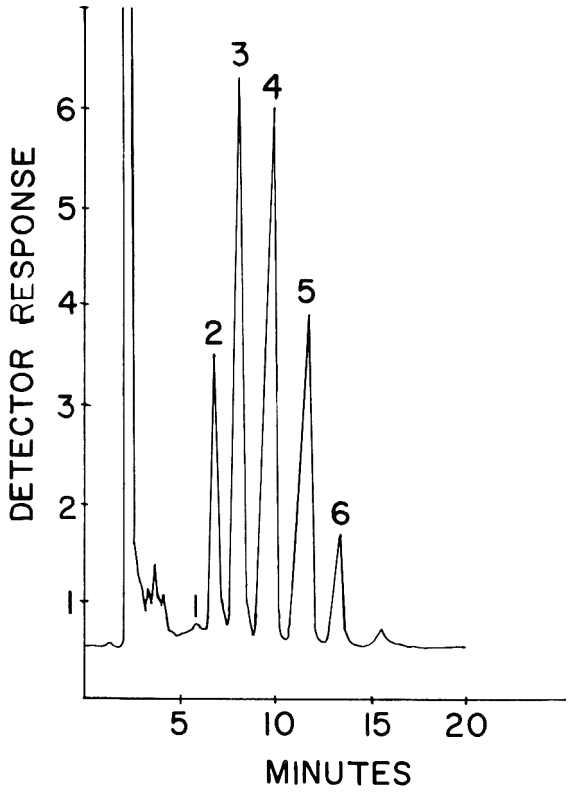

Figure 1. Liquid chromatogram of oil from sesame seeds. See text for peak description.

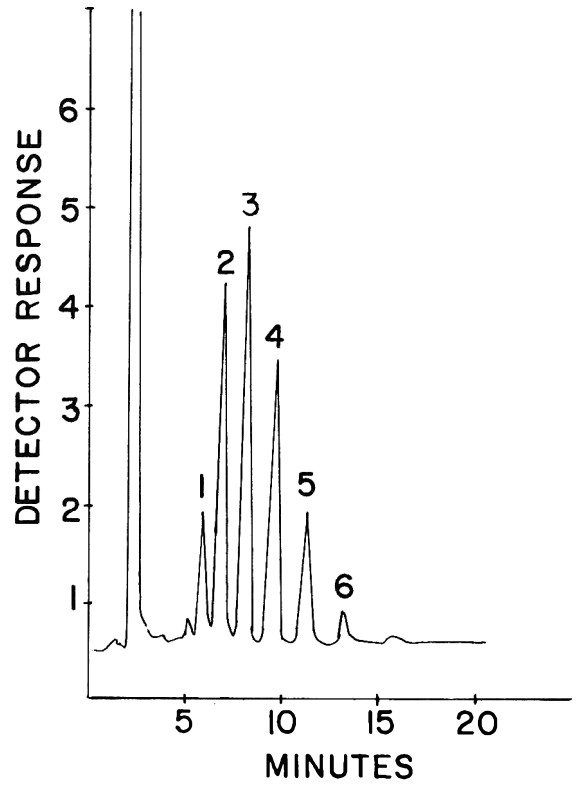

Figure 2. Liquid chromatogram of oil from soybeans. See text for peak description.

Table 1. Peak ratio (C_{42}/C_{46}) of sesame oil and soybean oil

Oil	Detns	Mean	SD	RSD, %	Range
Sesame	10	0.496	0.036	7.2	0.44–0.55
Soybean	9	1.253	0.039	3.1	1.21–1.34

triglycerides trimyristin (C_{42}) and tripalmitin (C_{48}) and by comparison with the pattern observed for olive oil and the ECN assignment by Supelco (8). Thus, peaks numbered 1, 2, 3, etc., correspond to ECN values of 40, 42, 44, etc., respectively.

For a pure oil, the ratio of any 2 peak heights in the same chromatogram is essentially constant and reproducible in successive determinations. Table 1 presents the results of multiple determinations for one ratio of peak heights for sesame and soybean oils.

When various known mixtures of sesame and soybean oils are chromatographed and the ratios of the heights of various pairs of peaks are plotted against the volume percent of either oil, the ratio of the heights of one pair of peaks, peak 2 (C_{42}) to peak 4 (C_{46}), is found to increase linearly with the percent of the adulterant (soybean oil) present. The average correlation coefficient for 6 plots obtained randomly over a 6 month period is 99% with a relative standard deviation of less than 2.5%. Likewise, the relative standard deviation obtained for 4 determinations of a calibration mixture or a sample is also less than 2.5%.

Although a larger relative standard deviation is observed for the C_{42}/C_{46} peak ratio obtained for the standard (pure) sesame oil than that obtained for the standard (pure) soybean oil, the range was not exceeded in those samples of importations which were shown by conventional fatty acid methyl ester analysis to be unadulterated. The chromatogram of the standard (pure) sesame oil did not appear to change significantly during the period of this investigation (6 months).

In contrast, although the C_{42}/C_{46} peak ratio is more consistent in the oil extracted from soybeans than in the oil extracted from sesame seeds, the chromatogram of the soybean oil does show some changes 3 months after extraction. The chromatogram of the freshly extracted soybean oil (Figure 2), indicates that all major triglycerides elute after 5 min. Trace components which elute in less than 4.5 min increase in concentration with time and become minor components, comparable in concentration to those of the peak preceding peak 1 (C_{40}). This minor change in the chromatogram does not alter the C_{42}/C_{46} peak height ratio significantly, but, to avoid any adverse effects, it is recommended that a new soybean extract be prepared every 3 months.

It should be noted, in Figure 1, that a peak corresponding to peak 1 (C_{40}) is essentially absent in the extracted (pure) sesame oil. Therefore, the presence of a significant C_{40} peak in an unknown sample is an indication that the sesame oil has been adulterated with another oil.

While soybean oil is frequently found as an adulterant in imported sesame oil, other oils have also been used, and the technique described can be used for other oils or even mixtures of more than 2 oils. The quantitative composition of mixtures containing 3 oils cannot be obtained in the same way that is possible with a 2 oil mixture; however, the chromatogram of an unknown oil may provide sufficient information to suggest the identity of the oils present and, by trial and error, to devise a composition that would duplicate the chromatogram of the unknown sample. In the chromatographic system used here for naturally occurring triglyceride mixtures, there is a high probability that 2 mixtures are identical when their components are observed to have identical retention times and relative concentration (peak height).

As an example, a sample from a commercial importation claimed to be sesame oil was chromatographed (Figure 3). Four major peaks are observed to elute between 10 and 20

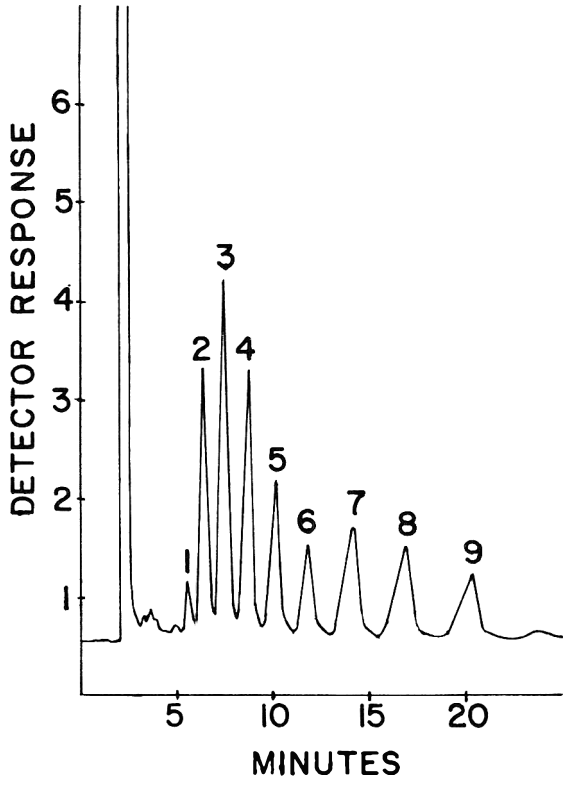

Figure 3. Liquid chromatogram of imported "sesame oil." See text for peak description.

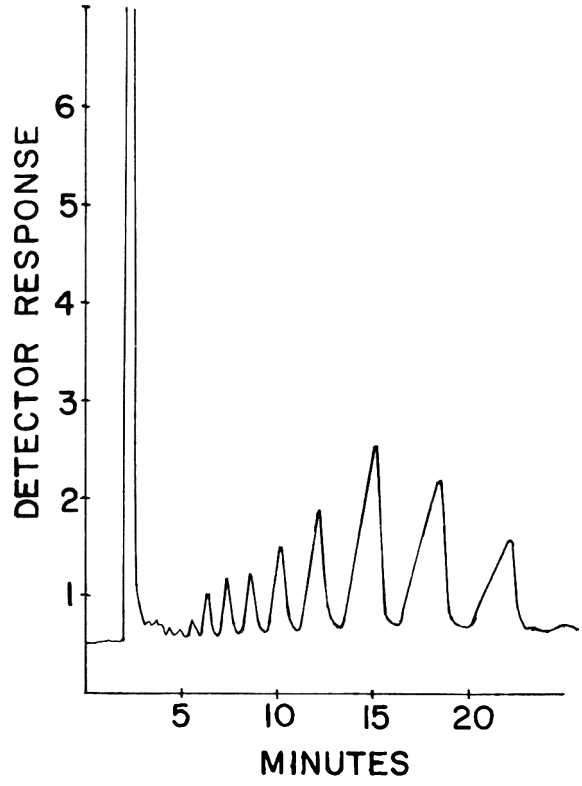

Figure 4. Liquid chromatogram of commercial rapeseed oil. See text for peak description.

min, suggesting that rapeseed oil (Figure 4), which is characterized by several late-eluting (long chain) major triglycerides, is present. It was noted, however, that the retention times of the late-eluting peaks averaged about 1 min shorter for the sample (importation) than for the pure rapeseed oil. The presence of rapeseed oil, despite the observed difference in retention times, was supported by LC of 4 known mixtures of sesame oil and rapeseed oil that contained increasing amounts of the sesame oil at volume ratios of 25:75, 40:60, 50:50, and 65:35. The chromatograms demonstrate that the retention times of the late-eluting triglycerides shortened as the concentration of the other oil (sesame oil) increased. For a 65% sesame oil mixture, the last major peak for pure rapeseed oil was observed at 21 min rather than 22 min.

Because the C_{42}/C_{45} peak ratio remained constant at 0.5 for the 4 known binary mixtures, and the importation had a peak ratio of 1.0, the presence of an additional adulterant was indicated. The high C_{42}/C_{46} peak ratio and the presence of a significant C_{40} peak (peak 1) suggested that the additional adulterant was soybean oil.

Composition of the importation was confirmed by preparing a mixture of the 2 oils, sesame and soybean, to reproduce the C_{42}/C_{46} ratio of 1.0 and then adding sufficient rapeseed oil to reproduce the chromatogram of the importation. A known mixture of sesame oil, soybean oil, and rapeseed oil, in the ratio of 15:45:40 by volume, resulted in a chromatogram considered identical to the chromatogram of the importation.

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TOBACCO

Liquid Chromatographic Determination of Phenol and Cresols in Total Particulate Matter of Cigarette Smoke

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The phenol and cresol contents of the total particulate matter of tobacco smoke can be conveniently determined. A simple extraction procedure using 1N base is followed by partial neutralization with glacial acetic acid and elution of hydroxybenzenes from an end-capped reverse phase liquid chromatographic column. The procedure is rapid (20 min/sample after the initial extraction), precise (± 10% relative standard deviation), accurate, and convenient. Normally, 12 or fewer cigarettes are needed for a single measurement.

Phenolic constituents, as a compound class, are believed to contribute to both the flavor and aroma (1) and the biological activity (2, 3) of tobacco smoke. In general, the concentrations of phenol and its derivatives in the smoke of filtered cigarettes tend to be lower than for unfiltered cigarettes because phenols are selectively removed from the smoke by the cellulose acetate used in most cigarette filters (4). However, important quantities of phenols remain in the mainstream total particulate matter (TPM) (5). Few quantitative data have been reported concerning the phenol levels in the smoke of modern commercial cigarettes, despite the increasing trend toward highly engineered, ventilated filters. The one exception is the recent work of Schlotzhauer and Chortyk (6), which reports values for phenol, catechol, and hydroquinone deliveries for one ultra low-tar U.S. commercial brand.

The classical determination of phenol and its methyl-substituted derivatives in cigarette smoke requires lengthy and laborious extraction procedures before final determination by gas chromatography (GC) (6–10). More recently, gel permeation chromatography has been used to purify the smoke extract, which is subsequently reacted with silylating reagents before final determination by open-tubular capillary column GC (11). Although that approach yields more information concerning levels of mono- and dihydroxybenzenes, the overall procedure is still laborious and unsuitable for routine use. Many of the reported studies require the collection of large amounts of cigarette smoke condensate (CSC) or pyrolysate in cold traps to obtain samples for analysis. This practice places a limitation on the inherent accuracy of the final determination. First, it is difficult to reproducibly and quantitatively collect CSC (12), and, second, the apparent phenol concentrations in condensate are typically 20–50% lower than those in the TPM-derived tar from identical cigarettes (8). This is presumably because phenol, a relatively volatile species, is lost in either the sample collection or work-up procedures. Trimethyl silylation of the more reproducibly generated TPM, followed by GC determination on packed (13) or open tubular capillary (6) columns, seems to be more successful.

We report here a quantitative procedure for determination of phenol and cresols in the total particulate matter of mainstream cigarette smoke, based on a modification of a liquid chromatographic (LC) procedure for phenols in synthetic fuel samples (14). Extraction and sample work-up are straightforward, and the overall method is sufficiently rapid to permit convenient processing of a large number of samples per day. The deliveries of phenols for both reference cigarettes and representative low-, medium-, and high-tar commercial cigarettes are reported.

METHOD

Apparatus

(a) *Liquid chromatograph.*—Single LDC Model 396 Simplex minipump (Riviera Beach, FL), Rheodyne Model 7120 injection valve or equivalent with 20 μL loop (Cotati, CA), Perkin-Elmer Model 203 fluorescence spectrophotometer (Norwalk, CT), and reverse phase C18 guard column (Brownlee Labs, Santa Clara, CA). Use stripchart recorder for readout.

(b) *Smoking device.*—Phipps and Bird (Richmond, VA) 20-port smoking machine, or equivalent.

(c) *Liquid scintillation counter.*—Tri-Carb liquid scintillation counter, Model C-2425 (Packard Instrument Co., Downers Grove, IL).

Reagents

(a) *Sodium hydroxide (1N), sodium acetate, and glacial acetic acid.*—Reagent grade, use as received.

(b) *Solvents.*—Acetonitrile, hexane, and water (distilled in glass, Burdick & Jackson Laboratories, Inc., Muskegon, MI).

(c) *Standard phenols.*—Phenol, *o*-cresol, *m*-cresol, *p*-cresol, resorcinol, and 3,4-dimethylphenol purchased from Aldrich Chemical Co. (Milwaukee, WI), 99+% purity. Use as received.

(d) *Standard solutions.*—Dissolve standard phenols in acetonitrile for final concentrations of 1, 4, 10, and 20 μg/mL. Refrigerate all standards; solutions are stable at least 2 months.

(e) *Tracer.*—Carbon-14-labeled *m*-cresol (Methyl C-14, New England Nuclear, Boston, MA; not routinely available) dissolved in hexane to yield measured activity of 3.39×10^7 disintegrations per min/mL (dpm/mL). Specific activity was 7.9 mCi/mmole, and mass/volume concentration was 210 μg *m*-cresol/mL.

(f) *Scintillation cocktail.*—Aquasol-2® universal liquid scintillation cocktail (New England Nuclear).

Cigarettes

Kentucky Reference 1R1, 2R1, and 1R4F cigarettes were obtained from the Tobacco and Health Research Institute (University of Kentucky, Lexington, KY). Code 32 cigarettes

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were obtained through the National Cancer Institute's Smoking and Health Program. The 8 commercial cigarette brands used in this study were purchased locally in June 1983.

Procedure

If cigarettes are not to be used immediately, store frozen at −22°C until needed. Before smoke generation and subsequent analysis, thaw and open cigarette packs, and place cigarettes in an environmental chamber maintained at standard conditions (15) of 60 ± 2% relative humidity at 24 ± 1°C. Condition cigarettes for at least 48 h. Select cigarettes at random for smoking.

Smoke sufficient cigarettes to yield ca 100 mg TPM on a standard Cambridge filter assembly (16) in room maintained at 60% relative humidity and 24°C. Use standard smoking conditions (15) of 35 ± 0.2 mL puff volume, 2 ± 0.2 s puff duration, and 1 puff per 60 ± 1 s frequency, to 23 mm butt length for nonfilter cigarettes or to 3 mm ahead of filter overwrap for filter cigarettes.

Begin extraction of every Cambridge filter pad not more than 1 h after smoking is complete. Spike each pad with 10 μL *m*-cresol tracer solution, and immediately immerse pad in 20 mL scintillation vial containing 10 mL 1N NaOH solution. Shake vial briskly with wrist-action shaker for 20 min, then let vial stand without shaking for 2 h. Dilute 500 μL aliquot of extract with equal volume of buffered mobile phase (see *Liquid Chromatography*), and partially neutralize solution with 30 μL glacial acetic acid. Analyze an aliquot of this final solution by LC.

Liquid Chromatography

Perform LC analysis as follows: analytical column, Alltech Associates RSIL-C18-HL, 25 cm × 4.6 mm id (Deerfield, IL). Mobile phase, acetonitrile–water (40% v/v), buffered to pH 4.6 with glacial acetic acid and sodium acetate, flow rate 1.4 mL/min. Set wavelengths of fluorescence detector to 266 nm (excitation) and 300 nm (emission). Inject 20 μL sample onto LC column. Perform quantitation by manual or electronic measurement of peak height.

Recovery Measurement

Mix 100 μL of final diluted extract with 10 mL scintillation cocktail and 1 mL water. Obtain dpm/mL count by using carbon-14 channel of liquid scintillation counter. Count samples 3 times for 10 min at room temperature. Correct all sample counts for scintillation quenching with Automatic External Standard option of instrument.

Calculations

Fit peak heights of phenols standard peaks (from 20, 10, 4, and 1 μg/mL mixed standard solutions) to standard linear regression equation to obtain $f(h)$, concentration of compound as function of peak height (correlation coefficient typically exceeds 0.995). Calculate hydroxybenzene delivery, C, of smoked cigarettes as follows:

$$C,\ \mu g/cig. = \frac{f(h)\ \mu g/mL \times 1\ mL \times 10\ mL}{n\ cigs \times 0.5\ mL \times Y}$$

$$\sim 20\, f(h)/n$$

where n is number of cigarettes smoked and Y is recovery of *m*-cresol tracer.

Results and Discussion

The optimum extraction time for Cambridge filter pads was determined by shaking a test pad briskly with 1N sodium hydroxide solution in a scintillation vial, letting the vial stand undisturbed for 24 h, and periodically withdrawing and analyzing aliquots. Extraction was virtually complete in 1 h, and showed no significant improvement after 2-, 4-, 6-, 8-, or 12-h extraction times. A nominal extraction period of 2 h was selected as both sufficient and convenient.

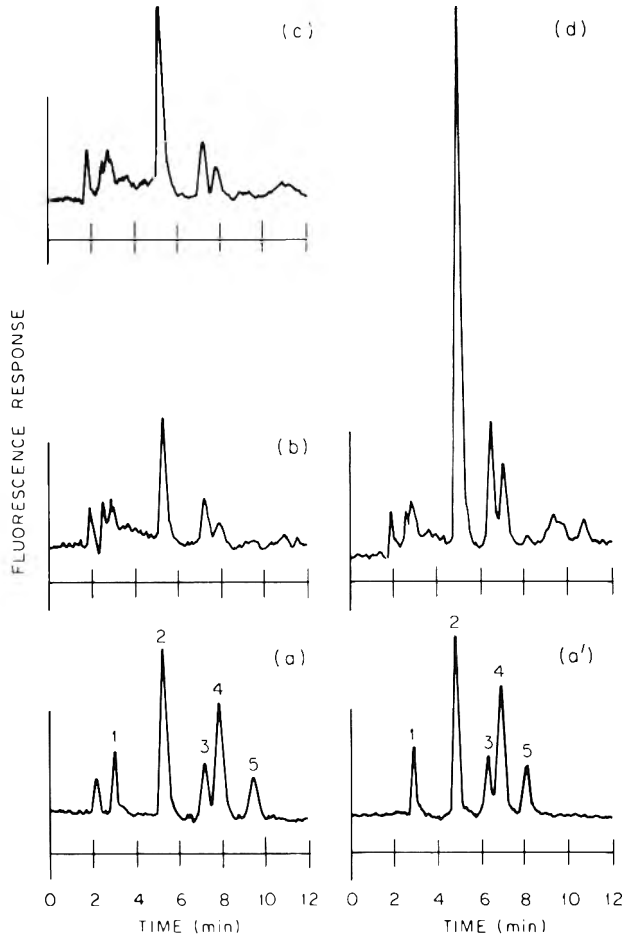

Figure 1. Phenols and cresols present in 3 "most popular" cigarette brands. Labeled peaks: resorcinol (1), phenol (2), *m*- + *p*-cresol (3), *o*-cresol (4), 3,4-dimethylphenol (5).

Chromatograms: (a) mixed phenols and cresols standard, 4 μg/mL per component, analyzed on day 1; (b) Code 550 (low-tar cigarette), analyzed on day 1, 9 cigarettes per pad; (c) Code 549 (medium-tar cigarette), analyzed on day 1, 5 cigarettes per pad; (a') mixed phenols and cresols standard, 4 μg/mL per component, analyzed on day 2; (d) Code 546 (high-tar cigarette), analyzed on day 2.

The optimum extraction time can also be determined by monitoring the recovery of the *m*-cresol radiolabeled tracer. This tracer was selected because its volatility and extraction behavior is intermediate between that of phenol, the major volatile phenolic constituent, and the dimethylphenols, which are minor and less volatile constituents. Hence, the recovery of *m*-cresol should be applicable to phenol, the 3 cresols, and most of the dimethylphenols. In most cases, *m*-cresol tracer was quantitatively recovered (>95%), thereby simplifying the equation. The mass of the radiolabeled tracer is negligible with respect to the mass of native *m*- or *p*-cresol observed, and may be omitted from the calcualtions for these compounds.

The analytical method cannot distinguish *p*-cresol and *m*-cresol, and is unsuitable for determining catechol. The *m*- and *p*-cresols co-elute, as shown in Figure 1, and could not be determined as independent species. Attempts to resolve these 2 isomers by modifying the chromatographic program were unsuccessful, so *m*- and *p*-cresol have been reported as though the peak were entirely *m*-cresol. This choice was

Table 1. Phenol and cresol deliveries for reference and experimental cigarettes

Sample	n^a	Method	Delivery,[b] μg/cig		
			Phenol	*m*- + *p*-Cresol[c]	o-Cresol
1R1[d]	4	LC	136 ± 6	88 ± 4	28 ± 2
1R1		GC[e]	120	69	27
2R1[f]	4	LC	130 ± 14	93 ± 7	34 ± 2
1R4F[d]	6	LC	16 ± 1	20 ± 1	6 ± 1
32[f]	4	LC	77 ± 7	56 ± 5	19 ± 1
32	1	GC[g]	70 ± 5	45 ± 3	15 ± 2

[a] Number of cigarettes smoked per pad.
[b] Mean ± 1 SD, 3 pads per measurement.
[c] Reported as entirely *m*-cresol.
[d] Kentucky reference cigarette, University of Kentucky, Lexington, KY.
[e] No statistical data available; data taken from ref. 13.
[f] National Cancer Institute Series IV Standard Experimental Blend.
[g] Mean ± 1 SD, based on 8 pads, 1 cigarette per pad.

Table 2. Recovery of phenol and cresols added to pads: summary of validation data, standard addition method[a]

Condition	Delivery,[b] μg/pad		
	Phenol	*m*- or *p*-Cresol[c]	o-Cresol
No spike added (u_1), mean ± 1 SD, 3 trials	62 ± 5	80 ± 5	23 ± 4
10 μg of each compound added (u_2), mean ± 1 SD, 3 trials	82 ± 1	98 ± 4	40 ± 2
t_{calcd}[d]	3.44*	2.33	2.61
Criterion: $t_{0.05}(4)$	2.776	2.776	2.776
No spike added	55	74	24
20 μg of each compound added	77	90	47
Spike recovered	22	16	23
No spike added	55	74	24
30 μg of each compound added	88	109	58
Spike recovered	33	35	34

[a] A pad containing TPM from six 1RF4 cigarettes was analyzed before and after spiking with 10, 20, or 30 μg of phenol, *m*-cresol, and o-cresol.
[b] Average of duplicate trials unless stated otherwise.
[c] Reported as entirely *m*-cresol.
[d] Tests null hypothesis H_0: $u_2 - u_1 - 10 = 0$.
*Signif cant difference at 5% significance level; not significant at 1% level.

made because the fluorescence response of *m*-cresol is about half that of *p*-cresol. For this reason, the values presented are probably somewhat conservative. The determination of catechol, a weak co-carcinogen (17), was not possible because this compound degrades quickly in the presence of 1M sodium hydroxide solution.

Validation Studies

The analytical method was verified both by the method of standard addition and by comparing results with those obtained independently by GC (13). Table 1 gives the deliveries of phenol and cresols of 4 reference cigarettes determined by LC, and compares the results with available GC measurements of silylated TPM extracts (13). Agreement between the present LC and the GC data was considered acceptable. (The 2R1 cigarette is a remake of the 1R1, using tobacco from a different crop year. The LC results for the 2R1 are quite similar, as expected, to those determined by LC and the GC procedure for the 1R1.) The nominal precision of the LC method was ±10% relative standard deviation, based on triplicate pad samples.

In tests using the method of standard addition, a 1R4F pad containing the TPM from 6 cigarettes was spiked with 10, 20, or 30 μg phenol, *m*-cresol, and *o*-cresol, and analyzed as before. The data in Table 2 show that the addition of 20 or 30 μg of a selected component per pad was readily detected and reliably quantitated, but that the addition of 10 μg to the pad was not.

The difference between the means of both the spiked (10 μg/pad) and unspiked pads was tested statistically using a 2-tailed Student's *t*-test. The results showed that, within the observed standard deviations of the 2 data sets, the difference of 10 μg/pad had indeed been observed at the 5% significance level. It appears that a 10 μg/pad increase approximated the detection limit of the LC system (0.3 μg/mL for phenol, 1.2 μg/mL for *m*-cresol, and 0.7 μg/mL for *o*-cresol at $S/N \sim 2$), and thus the increases in concentration were not easy to measure. Overall, the method of standard addition verified the accuracy of the LC procedure above the 10 μg/pad concentration level.

Volatilization of Phenol and the Cresols

A TPM sample mass of approximately 100 mg was used throughout this work because it usually provided a sufficient quantity of phenols for easy measurement, and because, in most cases, such a mass could be generated with a small number of cigarettes. In the special case of ultra low-tar cigarettes, which typically deliver 1–5 mg TPM/cigarette, many more cigarettes (up to 50) might be needed for a proper sample. The need for such a large number of cigarettes raised

Table 3. Volatilization study for phenol and the cresols

Condition	Delivery, μg/pad		
	Phenol	*m*- + *p*-Cresol[a]	o-Cresol
No. smoked:			
6 cigarettes (u_1)	62 ± 5[b]	80 ± 5	23 ± 4
12 cigarettes (u_2)	113 ± 19	135 ± 28	39 ± 8
t_{calcd}[c]	0.59	0.99	0.88
Criterion: $t_{0.05}(4)$	2.776	2.776	2.776
6 cigarettes (u_1)	62 ± 5	80 ± 5	23 ± 4
18 cigarettes (u_3)	171 ± 14	181 ± 6	48 ± 6
t_{calcd}[d]	0.77	5.39**	2.38
Criterion: $t_{0.05}(4)$	2.776	2.776	2.776

[a] Reported as entirely *m*-cresol
[b] Mean ± 1 SD, based on 3 pads.
[c] Tests null hypothesis H_0: $(u_2) - 2(u_1) = 0$.
[d] Tests null hypothesis H_0: $(u_3) - 3(u_1) = 0$.
** Significant difference at 1% significance level.

Table 4. Deliveries of phenol and cresols for selected U.S. commercial cigarette brands

ORNL code	Tar delivery,[a] mg/cig.	Cigarette type[b]	*n*[c]	Delivery,[d] μg/cig.		
				Phenol	*m*- + *p*-Cresol[e]	o-Cresol
552	24.3	85, nf	4	76 ± 2	61 ± 4	21 ± 0.1
546	20.1	70, nf	4	61 ± 4	45 ± 3	16 ± 1
548	16.9	85, f	5	21 ± 2	22 ± 2	8 ± 1
553	12.4	85, f	6	16 ± 1	16 ± 1	5 ± 0.1
549	16.8	100, f	5	16 ± 3	16 ± 2	4 ± 0.3
545	9.0	85, f	9	9 ± 1	11 ± 1	3 ± 0.3
550	8.2	85, f	9	6 ± 1	8 ± 1	2 ± 0.4
558	4.6	85, f	30	10 ± 1	9 ± 1	2 ± 1

[a] From Federal Trade Commission Report, ref. 18.
[b] All cigarettes are soft-pack. Length of cigarette given in mm.
nf = nonfilter, f = filter.
[c] Number of cigarettes per pad.
[d] Mean ± 1 SD, triplicate pad samples.
[e] Reported as entirely *m*-cresol.

the question of whether phenol and/or the cresols would be slowly volatilized from the Cambridge pad, based on the time needed to generate the sample TPM (several hours), the steady passage of air through the pad during cigarette puffing, and the vapor pressure of these species.

This question was examined by smoking 6, 12, and 18 1R4F cigarettes (tar delivery 9 mg/cigarette) on 3 Cambridge filter pads each (total of 9 pads) over periods of 1, 2, and 3 h, respectively. If no volatilization had occurred, the phenol and cresols present after 12 and 18 cigarettes should be exactly 2 and 3 times that obtained from 6 cigarettes. These hypotheses were tested using a 2-tailed Student's *t*-test at the 5% significance level.

Table 3 summarizes the data in the volatilization study. The phenol and cresol means for 12 cigarettes were not significantly different at the 5% significance level from twice that for 6 cigarettes; hence, a dozen cigarettes could be smoked without concern for volatilizaton. When 18 cigarettes were smoked, the means for phenol and *o*-cresol were not significantly different at the 5% significance level from 3 times that for 6 cigarettes. In contrast, the mean for *m*- and *p*-cresol (determined as *m*-cresol) was significantly different at even the 1% significance level from 3 times that for 6 cigarettes. Hence, the maximum number of cigarettes of this tar delivery level which should be smoked is probably about 12, which is sufficient to cover the high-, medium-, and low-tar cigarettes easily. Data presented later in this paper show that phenol deliveries from even ultra low-tar cigarettes can be determined precisely within these boundary conditions.

Application to Commercial Cigarettes

Figure 1 compares sample chromatograms for phenols from the TPM of high-, medium-, and low-tar cigarettes with each other and with a 4 μg/mL mixed phenol and cresol standard. The profiles are usually quite similar, except for the relative concentrations of each peak. It should be noted that different numbers of cigarettes were used to produce these chromatograms. Low-tar cigarettes required 9 cigarettes, high-tar cigarettes only 4. In contrast, the phenol delivery, expressed as mass per cigarette, varied by roughly an order of magnitude.

Table 4 reports the phenol and cresol deliveries of 8 popular commercial cigarette brands, ranging from high- to ultra low-tar delivery. Each measurement typically exhibited a precision of about ± 10% relative standard deviation, and was determined using triplicate Cambridge filter pads and smoking the stated number of cigarettes (4–9) per pad. As expected, the 2 nonfilter varieties had the highest deliveries of phenol and cresols per unit tar. The selective removal of phenols from particulate matter by cellulose acetate filters has been reported previously (4). The deliveries for Code 558, an ultra low-tar cigarette, may be biased low because of the large number of cigarettes required for the generation of a sufficient

smoke sample. However, the level of phenol is in reasonably good agreement with a value reported for another ultra low-tar cigarette (6), although because the tar deliveries of the 2 cigarettes are different, there is no reason to expect that the phenol deliveries should be the same.

Conclusion

The measurement of phenol, *m*- plus *p*-cresol, and *o*-cresol in the TPM of cigarette smoke is greatly simplified by using an LC procedure that uses both a buffered eluant and a selective fluorescent detector. Approximately 15–20 samples may be readily processed per working day, allowing for LC of 4 standards at the beginning of the day, 20 min chromatographic time per sample, and either a 2-h or overnight extraction.

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DRUGS

Separation and Analysis of Pilocarpine, Isopilocarpine, and Pilocarpic Acid by Reverse Phase Liquid Chromatography: Collaborative Study

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A method for separating and determining pilocarpine and 2 degradation products was developed and subjected to collaborative study. Pilocarpine, isopilocarpine, and pilocarpic acid were isolated on a reverse phase liquid chromatographic phenyl bonded column and detected by UV spectrophotometry at 220 nm. Nine collaborators received commercial samples labeled to contain 2, 1, and 0.5% pilocarpine and a 2% practice sample. The collaborative results for pilocarpine were excellent; coefficients of variation ranged from 3.20 to 4.10%. The method was adopted official first action for determination of the active component, pilocarpine, in the presence of isopilocarpine and pilocarpic acid. Although quantitative results for the degradation products were not as good, the method is suitable as a limits test for these substances.

Numerous methods have been devised in an attempt to separate pilocarpine and related compounds by liquid chromatography (LC). Urbanyi et al. (1) used Aminex A-7 cation-exchange resin with UV peak detection at 217 nm. Weber (2) attempted a 2-step modification but concluded that the procedure was inaccurate because of variation of the commercially available resins. Khalil (3), using a borate buffer (pH 9.2)–tetrahydrofuran (70 + 30) mobile phase, joined a μBondapak C_{18} column and a μBondapak CN column in series and detected pilocarpine at 254 nm. However, subsequent investigators observed that the mobile phase dissolved the silica base in the column. Mitra et al. (4) enhanced the C_{18} μBondapak column procedure by forming *p*-nitrobenzyl derivatives. Noordam et al (5) and O'Donnell et al. (6) separated pilocarpine and its degradation products by using a C_{18} column and a water–methanol (97 + 3) mobile phase containing 5% pH 2.5 phosphate buffer. Initially they used refractive index for detection but eventually settled on UV detection at 215–216 nm. Kennedy and McNamara (7) reported a reverse phase 10 μm μBondapak phenyl column procedure for separating pilocarpine, isopilocarpine, pilocarpic acid, and isopilocarpic acid. Two additional methods, normal phase (8) and reverse phase radial compression (9), have also been reported on the assay of pilocarpine. These allow quantitative determination of pilocarpine and isopilocarpine but not pilocarpic acid.

In his 1981 General Referee Report, Smith (10) referred to a method developed by Wainer for determining pilocarpine, isopilocarpine, and pilocarpic acid with a reverse phase phenyl LC column and a water–acetonitrile (97 + 3) mobile phase. We have conducted a collaborative study based on this method.

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This report of the Associate Referee, I. W. Wainer, was presented at the 97th Annual International Meeting of the AOAC, Oct. 3–6, 1983, at Washington, DC.

The recommendations of the Associate Referee were approved by the General Referee and by Committee B and were adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) **67,** March issue.

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Collaborative Study

Each collaborator received a solid reference sample of pilocarpine nitrate, a system suitability solution, one 2% practice sample solution (sample C), and 3 commercial sample solutions consisting of 2, 1, and 0.5% pilocarpine nitrate, plus the following ingredients: Pilocarpine 2% (generic): 0.5% methyl cellulose, 0.4% boric acid, 0.01% benzalkonium chloride, and 0.5% disodium edetate. Pilocarpine 1% (generic): same as 2% solution. Commercial brand 0.5%: 1.4% polyvinyl alcohol, 0.5% chlorobutanol, 0.5% menthol, 0.5% camphor, 0.5% phenol, and 0.5% eucalyptol. These additives account for the additional peaks in the chromatogram. Collaborators were instructed to prepare solutions for LC as follows: Sample D, 2% solution, dilute 1 mL to 200 mL; sample E, 1% solution, dilute 1 mL to 100 mL; sample F, 0.5% solution, dilute 2 mL to 100 mL. Each collaborator was asked to make 3 injections of each sample solution. Nine collaborators completed the study.

The practice solution and the 3 collaborative solutions were known from previous analyses in this laboratory to contain isopilocarpine and pilocarpic acid. Table 1 reports the percentage composition for 7 brands of pilocarpine ophthalmic solutions as originally determined by this laboratory.

Pilocarpine, Isopilocarpine, and Pilocarpic Acid in Drugs Liquid Chromatographic Method First Action

Principle

Pilocarpine is detd by LC, using acidified phosphate buffer–CH_3CN (97 + 3) mobile phase, reverse phase phenyl bonded column, and detection at 220 nm.

Apparatus

(a) *Liquid chromatograph.*—Equipped with 7000 psi injection valve with 10 μL injection loop and printer-plotter; UV-visible detector set at 220 nm, 0.04 AUFS, time const set at 4 s; data integration system with peak width set to peak threshold ratio of 1:60 (Spectra-Physics Model 8000 or appropriate settings for equiv. chromatgc data system).

(b) *Liquid chromatographic column.*—10 μm reverse phase phenyl bonded column, 30 cm × 3.9 mm id (Waters Associates, Inc.).

Reagents

(a) *Mobile phase.*—UV quality LC grade H_2O and CH_3CN (97 + 3). Add KH_2PO_4 to make 5% soln. Adjust pH to 2.5 ± 0.1, using 85% H_3PO_4. Filter soln thru 5 μm mixed cellulose acetate and nitrate filter.

(b) *Std soln.*—Dry USP Ref. Std Pilocarpine Nitrate Salt 2 h at 105°. Accurately weigh 10 mg into 100 mL vol. flask. Dissolve and dil. to vol. with mobile phase. Filter thru 5 μm mixed cellulose acetate and nitrate filter. Check std soln daily

Table 1. Original pilocarpine commercial ophthalmic solutions tested in Associate Referee's laboratory

Sample	Pilocarpine, %	Pilocarpic acid, %	Isopilocarpine, %
Brand A 2%, HCl[a]	96	4.0	0.21
Brand B 2%, HCl[b]	92	7.3	0.32
Pilocarpine HCl 2%	96	3.8	0.20
Brand C 2%, nitrite[c]	93	7.1	0.35
Brand D 1%, nitrite[c]	93	5.1	0.65
Brand E 1%, HCl[a]	94	5.1	0.64
Brand F 0.5%, nitrite[c]	92	6.9	1.4

[a]With hydroxypropyl methylcellulose, benzalkonium chloride, and EDTA.
[b]With 0.5% hydroxypropyl methylcellulose and 0.01% benzalkonium chloride.
[c]With 1.4% polyvinyl alcohol, 0.5% chlorobutanol, menthol, camphor, phenol, and eucalyptol.

Table 2. Collaborative results for pilocarpine ophthalmic solutions (g/mL) by reverse phase liquid chromatographic method[a]

	Sample C			Sample D			Sample E			Sample F		
Coll.	P	I	A	P	I	A	P	I	A	P	I	A
1[b]	—	—	—	2.22	0.028	0.088	1.10	0.013	0.047	0.538	0.015	0.045
	—	—	—	2.18	0.027	0.087	1.10	0.013	0.046	0.535	0.016	0.043
	—	—	—	—	—	—	1.10	0.014	0.045	0.544	0.016	0.041
2	2.15	0.021	0.113	2.24	0.028	0.087	1.12	0.011	0.055	0.516	0.013	0.046
	2.16	0.029	0.116	2.23	0.027	0.076	1.12	0.013	0.031	0.523	0.015	0.040
	2.16	0.045	0.151	2.24	0.019	0.090	1.12	0.010	0.045	0.525	0.015	0.039
3	2.20	0.018	0.080	2.20	0.017	0.081	1.09	0.006	0.039	0.621[c]	0.012	0.035
	2.20	0.017	0.077	2.21	0.015	0.079	1.10	0.005	0.037	0.544	0.010	0.029
	2.20	0.017	0.079	2.20	0.015	0.077	1.10	0.006	0.038	0.540	0.010	0.027
4[b]	2.02	0.032	0.167	2.29	0.033	0.129	1.08	0.015	0.067	0.498	0.017	0.065[c]
	2.14	0.031	0.170	2.22	0.036	0.115	1.10	0.015	0.067	0.505	0.017	0.065[c]
	2.13	0.035	0.170	2.31	0.039	0.117	—	—	—	—	—	—
5	2.05	0.061	0.075	2.14	0.066	0.066	1.12	0.052[c]	0.047	0.541	0.039	0.023
	2.00	0.050	0.075	1.87[c]	0.114[c]	0.062	1.11	0.050[c]	0.033	0.502	0.026	0.032
	2.20	0.192	0.139	2.00	0.038	0.071	1.11	0.025	0.035	0.533	0.031	0.021
6	2.22	0.030	0.079	2.35	0.027	0.063	1.12	0.013	0.045	0.504	0.013	0.029
	2.21	0.032	0.079	2.34	0.031	0.063	1.09	0.014	0.039	0.490	0.013	0.027
	2.32	0.030	0.080	2.34	0.030	0.066	1.21	0.014	0.043	0.466	0.013	0.026
7	2.09	0.042	0.124	2.11	0.033	0.099	1.06	0.013	0.048	0.510	0.016	0.045
	2.09	0.049	0.124	2.12	0.038	0.091	1.05	0.015	0.048	0.499	0.016	0.045
	2.09	0.044	0.121	2.11	0.029	0.093	1.06	0.015	0.049	0.501	0.016	0.045
8	2.16	0.029	0.116	2.23	0.034	0.083	1.15	0.016	0.043	0.526	0.015	0.030
	2.15	0.031	0.112	2.23	0.030	0.082	1.18	0.014	0.042	0.528	0.014	0.028
	2.14	0.035	0.111	2.24	0.028	0.081	1.16	0.015	0.041	0.534	0.016	0.028
9	2.15	0.037	0.082	2.26	0.040	0.084	1.10	0.021	0.036	0.541	0.018	0.021
	2.14	0.038	0.086	2.25	0.047	0.080	1.10	0.015	0.034	0.546	0.017	0.023
	2.14	0.032	0.078	2.31	0.043	0.078	1.04	0.015	0.034	0.549	0.019	0.020
Mean	2.15	0.034	0.108	2.22	0.032	0.084	1.11	0.014	0.044	0.522	0.017	0.033
SD	0.069	0.011	0.032	0.082	0.011	0.017	0.038	0.004	0.009	0.021	0.006	0.009
CV	3.20	32.1	29.8	3.70	34.0	19.7	3.39	31.3	20.7	4.10	37.2	27.4

[a]Sample C, 2% practice sample; Sample D, 2% commercial solution; Sample E, 1% commercial solution; Sample F, 0.5% commercial solution. P, pilocarpine; I, isopilocarpine; A, pilocarpic acid.
[b]Dash indicates that value was not reported by collaborator.
[c]Value rejected as outlier; not included in total statistics.

by LC injection; prep. new std soln if pilocarpine concn, as detd by peak area, has changed >2%.

(c) *System suitability std soln.*—Dil. 10 mg pilocarpine and 1 mg isopilocarpine to 100 mL with mobile phase.

Preparation of Sample

Prep. soln contg 10 mg pilocarpine nitrate salt/100 mL (based on label concn) using mobile phase as diluent. Filter soln thru 5 μm mixed cellulose acetate and nitrate filters.

Liquid Chromatography Test

Equilibrate overnight at 26 ± 1° with mobile phase at flow rate of 0.3 mL/min. Circulate solv. continuously thruout duration of analyses, without interruption.

System suitability test.—Inject 10 μL aliquots of system suitability std soln into chromatgc column. Retention time for pilocarpine should be 50–54 min, and isopilocarpine, 45–49 min. Resolution factor, R_s, for pilocarpine/isopilocarpine should be ≥1.13.

Determination

Inject 10 μL aliquots of pilocarpine std soln and sample prepn in triplicate. Calc. quantity, in g/100 mL pilocarpine, in each sample by formula:

$$\text{g pilocarpine (as nitrate)/100 mL} = PA/PA' \times C \times D$$

where PA and PA' = area of pilocarpine peak for sample and std solns, resp.; C = g std/100 mL; D = diln factor of sample prepn. Altho not necessarily quant., isopilocarpine (retention time 45–49 min) and pilocarpic acid (retention time 33–38 min) may be estd by use of stds and similar calcns. When using different pilocarpine salts, use correction factor for different mol. wts: F = MW pilocarpine salt/MW pilocarpine

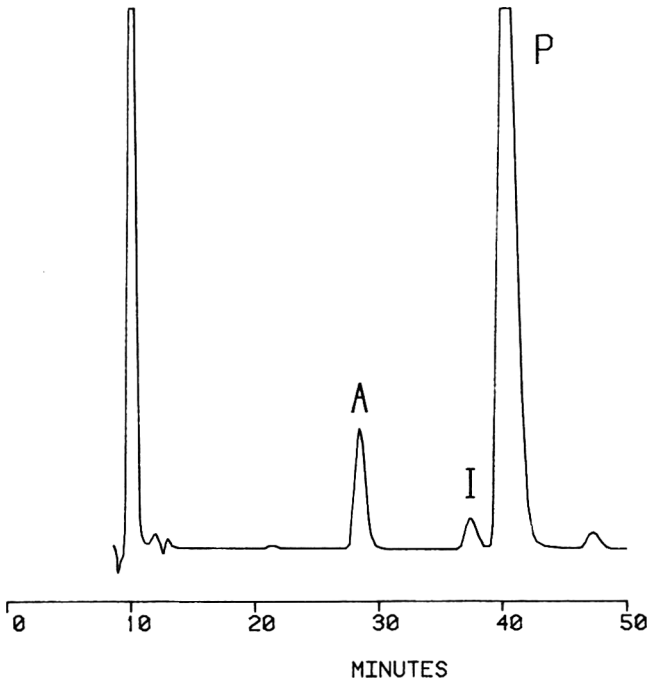

Figure 1. Sample chromatogram for separation of pilocarpine (P), isopilocarpine (I), and pilocarpic acid (A).

std (corrects for differences in absorptivity between pilocarpine and pilocarpic acid).

Results and Discussion

Table 2 shows collaborative results for pilocarpine ophthalmic solutions by reverse phase liquid chromatography. Figure 1 shows a sample chromatogram of pilocarpine and 2 degradation products.

The collaborators obtained proper R_s values and retention times for the system suitability test either directly or by minor modifications such as reduction of the acetonitrile concentration. The originally specified flow rate of 0.25 mL/min was unacceptable because most instruments used had settings of 0.2 or 0.3. We modified our procedure to specify a flow rate of 0.3 mL/min. Although 1 or 2 collaborators reported instrumental problems with the high salt buffer concentration, proper flushing of the system after use should alleviate this problem.

Collaborator 1 did not report data for sample C because of changes in instrument parameters between injections, and made only 2 injections for sample D. Collaborator 4 reported only 2 injections for samples E and F because of fluctuations in detector lamp energy, which necessitated making all analyses in one day.

Good statistical results for the major component, pilocarpine, support the method of analysis. Outliers were rejected on the basis of pooled results, not individual laboratories. Both Dixon's test and the less rigorous 2.5 D rule identified outliers. Substantial statistical improvement was obtained in this manner for the minor components, isopilocarpine and pilocarpic acid. The relatively high standard deviations and coefficients of variation which still remain for these components are due to low actual concentrations relative to pilocarpine. Coefficients of variation for pilocarpine ranged from 3.20 to 4.10%.

Recommendations

On the basis of collaborative study results, it is recommended that this method be adopted official first action for determination of pilocarpine in the presence of the decomposition products isopilocarpine and pilocarpic acid. Although the coefficients of variation for the decomposition products are high (Table 2), the method is also recommended as a test for limits for these materials.

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X-Ray Powder Diffraction Data for Selected Drugs: Furosemide, Hydrochlorothiazide, Naproxen, Naproxen Sodium, Propranolol Hydrochloride, and the Halopheniramine Maleates

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X-ray powder diffraction data for 9 commonly used drug substances are reported. The data for furosemide, hydrochlorothiazide, propranolol hydrochloride, dexchlorpheniramine maleate, and brompheniramine maleate have been indexed by reference to published crystal structure data. The racemic modifications of propranolol hydrochloride and brompheniramine maleate are shown to exist as racemic compounds rather than racemic mixtures.

The application of X-ray diffraction to the investigation of problems involving drugs has long been hindered by the lack of appropriate reference data for the dosage form of the drugs. Thus, studies of the relationship between polymorphism of a drug substance and its bioavailability, for example, are frequently carried out with only a limited characterization of the drug substance in the crystalline state. This paper represents the inception of a continuing program to increase the availability of reference quality diffraction data for drug substances. The series will focus primarily, but not exclusively, on those drugs which are the subjects of compendial monographs and which are, for the most part, also available as Reference Standards through the United States Pharmacopeial Convention (USP). The drug substances will, consequently, be investigated in the chemical form or forms (e.g., salt, ester, stereoisomer) which are the actual components of the dosage forms of the drugs. Furthermore, we propose to investigate primarily, although not exclusively, drug substances which are in widespread use.

An extensive file of powder diffraction data is published by the Joint Committee for Powder Diffraction Standards-International Centre for Diffraction Data (1). This file is designed for manual searching as well as either interactive or batch computer searching. (Information about various commercially available search-match programs may be obtained from JCPDS-International Centre for Diffraction Data, 1601 Park Lane, Swarthmore, PA 19081.) Through the most recent set (volume 33), this file contains data for the the identification of over 40 000 crystalline materials. For a variety of technical and historical reasons, most of the data in the file are for inorganic compounds. However, the present state of the art is such that reliable data can be obtained for most organic materials. In most cases, a knowledge of the *d*-spacings alone is sufficient to positively identify a crystalline material. The relative intensity associated with each *d*-spacing is of some use in distinguishing between nearly isomorphous materials and can be used for semiquantitative estimates of concentration as well.

For all the drug substances discussed herein, X-ray powder diffraction is not a required identity test. Its use is consistent, however, with the compendial standards for verification of the identity of a drug substance, which recognize that the compendial test(s), ". . . however specific, are not necessarily sufficient to establish proof of identity . . ." (2, p. 6).

Selection of Samples

Furosemide [$C_{12}H_{11}ClN_2O_5S$; 4-chloro-*N*-furfuryl-5-sulfamoylanthranilic acid; CAS-54-31-9] is a diuretic and antihypertensive drug (3, monograph 4161). It ranked sixth among the most frequently prescribed drugs in 1982 (4). The compendial monograph in USP XX (2) specifies infrared (IR) (KBr pellet) and ultraviolet (UV) absorption tests, plus a color reaction, for its identification.

Hydrochlorothiazide [$C_7H_8ClN_3O_4S_2$; 6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide; CAS-58-93-5] is a sulfamide diuretic of the benzothiadiazine family, and is commonly used both singly and in combination with other prescription drugs (3, monograph 4668). The compendial monograph in USP XX (2) specifies IR (KBr pellet) and UV absorption tests for its identification. In 1982, the most prescribed drug was a combination containing hydrochlorothiazide; 12 additional products containing hydrochlorothiazide were also among the 200 most prescribed drugs (4). In addition, it is the most commonly prescribed generic drug which is not an antibiotic.

Naproxen [$C_{14}H_{14}O_3$; (+)-6-methoxy-α-methyl-2-naphthaleneacetic acid; CAS-22204-53-1] is an anti-inflammatory, analgesic, and antipyretic drug (3, monograph 6245), which ranked twenty-third among the most frequently prescribed drugs in 1982 (4). It is also marketed as the sodium salt [naproxen sodium; $C_{14}H_{13}NaO_3$; (−)-sodium 2-(6-methoxy-2-naphthyl)propionate; CAS-26159-34-2], and is ranked among the 200 most prescribed drugs. A compendial monograph for naproxen, specifying only an IR (KBr pellet) test for identification, was first published in Addendum *a* to the First Supplement to USP XX (5). A compendial monograph for naproxen sodium was first published in Addendum *a* to the Third Supplement to USP XX (6), and also specifies only an IR (KBr pellet) identity test.

Propranolol hydrochloride [$C_{16}H_{22}ClNO_2$; 1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol hydrochloride; CAS-318-98-9] is a beta-adrenergic antagonist which is widely used in the treatment of conditions such as angina pectoris, cardiac arrhythmia, hypertension, and other cardiovascular disorders (3, monograph 7628). It ranks second among the most frequently prescribed drugs (4). The compendial monograph in USP XX (2) specifies IR (mineral oil mull) and UV absorption tests, plus a test for chloride ion, for its identification.

Brompheniramine maleate [$C_{16}H_{19}BrN_2 \cdot C_4H_4O_4$; 2-[*p*-bromo-α-[2-(dimethylamino)ethyl]benzyl]pyridine maleate (1:1)] is among the more frequently prescribed antihistaminics (3, monograph 1466; 4). It is used in drugs both as the racemate (CAS-980-71-2) and as the pure *d*-isomer [CAS-2391-03-9, dexbrompheniramine maleate (United States Adopted Name (USAN))]. The compendial monographs in USP XX (2) specify both an IR and a UV absorption test for the identification of both brompheniramine maleate and dexbrompheniramine maleate, but differ in specifying a KBr pellet for the former and a mineral oil mull for the latter. In the latter case, a determination of specific rotation is also included among the specifications.

Chlorpheniramine maleate [$C_{16}H_{19}ClN_2 \cdot C_4H_4O_4$; 2-[*p*-chloro-α-[2-(dimethylamino)ethyl]benzyl]pyridine maleate (1:1)] is also a commonly prescribed antihistaminic drug (3, monograph 2169; 4). Although it is not as frequently used as brompheniramine maleate, it is a component of numerous over-the-counter and prescription drugs. Both the racemate (CAS-113-92-8) and the pure *d*-isomer [CAS-2438-32-6, dexchlor-

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Table 1. X-ray powder diffraction data for furosemide, USP

2θ	d(Å)	I/I_1	hkl	2θ	d(Å)	I/I_1	hkl
6.017	14.68	100	010	25.561	3.482	1	−2,−3,2
10.843	8.15	4	001	26.221	3.396	8	1,−1,2; −1,−4,2
12.044	7.34	23	020	27.543	3.236	7	−2,4,0
13.025	6.79	3	0,−2,1	28.664	3.112	17	022; 211
14.207	6.229	12	011	29.226	3.053	8	230
17.050	5.196	1	−2,0,1	29.766	2.999	7	1,−4,2; −1,0,3
17.491	5.066	6	−1,2,1; 0,−3,1; 101	30.467	2.932	4	−1,3,2; 050; −2,−3,3
18.232	4.862	21	−2,−1,1	30.847	2.896	1	310; 0,−2,3
18.793	4.718	20	−2,1,0	31.549	2.833	8	0,−3,3
18.953	4.678	23	021	33.631	2.663	2	2,−5,1; −3,3,2
19.113	4.640	6	200	33.850	2.646	2	320; 2,−1,2
20.474	4.334	16	111; 0,−1,2; 1,−3,1	34.252	2.616	3	−2,−5,2; 3,−1,1
21.075	4.212	12	−2,−2,1	35.013	2.561	1	3,−3,1; −4,0,1; −4,1,2
21.356	4.157	30	210; −2,0,2	35.553	2.523	3	301
21.856	4.063	1	002	36.715	2.446	2	060; −2,−5,3
22.918	3.877	28	0,−3,2	37.475	2.398	2	−3,5,0; −4,3,1
23.198	3.831	6	−2,1,2	38.097	2.360	5	212; −4,2,0; 2,−6,1
23.400	3.799	3	−2,3,0	39.037	2.305	3	−4,3,2
24.800	3.587	75	−1,−4,1; 220	39.718	2.267	1	250
25.180	3.534	9	−2,−3,1				

Table 2. X-ray powder diffraction data for hydrochlorothiazide, USP

2θ	d(Å)	I/I_1	hkl	2θ	d(Å)	I/I_1	hkl
9.458	9.34	6	001	34.111	2.626	6	130
12.802	6.91	9	100	35.853	2.503	10	023
14.064	6.292	2	011	36.695	2.447	7	−3,0,2
16.507	5.366	80	110	37.395	2.403	8	113
18.630	4.759	44	101	37.776	2.380	3	202
19.051	4.655	100	002	38.196	2.354	1	−3,1,2
20.833	4.260	39	020	39.237	2.294	6	−3,0,3
21.354	4.158	30	111	40.098	2.247	1	014
21.714	4.089	4	012	40.659	2.217	1	−3,1,3
22.936	3.874	4	021	41.181	2.190	1	−2,3,2
24.538	3.625	28	120	41.802	2.1592	4	132
25.860	3.443	11	−2,0,2	42.543	2.1233	1	−3,2,2
26.280	3.388	3	−2,1,1	43.523	2.0777	2	222
26.721	3.333	1	102	43.944	2.0588	3	−3,0,4
27.902	3.195	14	210	44.464	2.0359	3	140
28.363	3.144	7	022	45.287	2.0008	1	−3,1,4
28.764	3.101	25	003	46.048	1.9695	3	203
30.787	2.902	4	201	46.789	1.9400	4	114
32.589	2.745	5	211	47.309	1.9199	2	213
32.950	2.716	1	031	48.431	1.8780	1	133
33.410	2.680	9	220	49.091	1.8543	1	321

pheniramine maleate (USAN)] are used as drug substances. The compendial monographs in USP XX (2) specify only an IR (mineral oil mull) test for the identification of chlorpheniramine maleate, but both IR (KBr pellet) and UV absorption tests for the identification of dexchlorpheniramine maleate. In the latter case, a determination of specific rotation is also included among the specifications.

Experimental Conditions

X-ray powder diffraction patterns were obtained with CuKα radiation (λ = 1.54051 Å) on a Philips goniometer. The X-ray tube was typically operated at 40 kV and 30 mA. Reference Standard materials were scanned at a speed of 0.25°/min (2θ), with a detector time constant of 2. Other samples were scanned at 1°/min, with a time constant of 0.5. A θ-compensating slit (7) maintained a constant area of illumination on the sample throughout the range of diffraction angles. A diffracted-beam monochromator (graphite 001) was used to remove the unwanted radiation. The detector was a NaI(Tl) scintillation counter, coupled to a pulse height analyzer.

The 2θ values from the chart recorder were measured to ±0.02° for each resolved peak. A linear correction was applied to the 2θ values, based on calibration of the instrument using Ag (a_0 = 4.08651 Å) or Si (NBS SRM640, a_0 = 5.43088 Å) as an external standard. The intensity of each diffracted line was measured by correcting the peak height from the strip chart recorder for background. A correction was applied to the intensity to allow for the effects of the θ-compensating slit (C. R. Hubbard, National Bureau of Standards, 1979).

Samples were prepared by loosely filling the well of an aluminum sample holder with the powder, covering it with a ground glass slide, and vibrating the holder to pack the sample. Commercial samples which were available only in limited amounts were sprinkled onto a glass slide which was lightly coated with petroleum jelly.

Melting points were determined on a Mettler HP52 hot stage microscope and are reported as uncorrected values. Each sample was placed on the hot stage at a temperature approximately 10°C below its melting point, and heated at a rate of 2°C/min. Under these conditions, samples of USP Sulfapyridine Reference Standard (Lot H) melted at 191.2–191.9°C, compared with the range 190.2–192.0°C specified by USP, and USP Acetanilid Reference Standard (Lot G) melted at 113.0–114.2°C, compared with the range 114.0–116.0°C specified by USP.

Origin of Samples

In each case, the primary sample examined was the current lot of the Reference Standard as distributed by USP. Where

Table 3. X-ray powder diffraction data for naproxen, USP

2θ	d(Å)	I/I_1
6.683	13.22	100
12.627	7.00	28
13.348	6.628	15
16.811	5.270	22
17.992	4.926	8
18.933	4.684	38
20.073	4.420	14
20.333	4.364	13
22.275	3.988	29
23.736	3.745	15
23.996	3.705	11
25.278	3.521	1
27.379	3.255	10
27.820	3.204	8
28.420	3.138	11
29.340	3.042	1
29.781	2.998	2
31.423	2.845	3
32.383	2.762	1
33.204	2.696	<1
33.745	2.654	2
34.885	2.570	2
35.105	2.554	2
36.507	2.459	<1
37.167	2.417	1
37.627	2.389	1
38.228	2.352	1
38.648	2.328	1
39.790	2.264	1
40.749	2.212	2
42.371	2.1315	<1
44.494	2.0346	1
45.534	1.9905	1
46.695	1.9437	1
49.757	1.8310	1

Table 4. X-ray powder diffraction data for naproxen sodium, USP

2θ	d(Å)	I/I_1
4.361	20.25	100
8.634	10.17	1
11.747	7.53	<1
13.048	6.78	19
15.610	5.672	<1
17.331	5.095	6
18.412	4.815	6
19.993	4.438	4
20.574	4.313	2
21.715	4.089	3
22.375	3.970	6
22.896	3.881	2
23.256	3.822	1
23.676	3.755	<1
24.437	3.640	2
25.278	3.521	1
25.898	3.438	<1
26.939	3.307	3
27.780	3.209	1
28.620	3.116	1
29.401	3.035	1
30.022	2.974	1
31.263	2.859	3
32.284	2.771	1
32.664	2.723	<1
35.005	2.561	<1
35.685	2.514	<1
36.007	2.492	<1
36.787	2.441	<1
37.107	2.421	<1
38.027	2.364	<1
39.088	2.303	1
41.091	2.190	<1
42.933	2.1049	<1
44.233	2.0460	<1
44.693	2.0260	<1
44.894	2.0174	<1
45.993	1.9717	<1
47.176	1.9250	<1
48.297	1.8829	<1
49.778	1.8303	<1
51.279	1.7802	<1
52.098	1.7541	<1
53.720	1.7049	<1
54.361	1.6863	<1

available, randomly selected samples of the same drug substance from one or more manufacturers were also examined. Except as noted below, each sample was dried in accordance with the compendial monograph. No further purification, such as by recrystallization, was attempted. Reference Standard samples were not ground as long as a large portion of the material as supplied passed through a 200 mesh sieve. The procedures required by USP XX do not include purification by recrystallization or any treatment other than grinding or sieving, as necessary.

The lots of USP Reference Standards used in this study were as follows: Furosemide (Lot G); Hydrochlorothiazide (Lot G); Naproxen (Lot F); Naproxen Sodium (Lot F); Propranolol Hydrochloride (Lot F); Brompheniramine Maleate (Lot G); Dexbrompheniramine Maleate (Lot G); Chlorpheniramine Maleate (Lot I); Dexchlorpheniramine Maleate (Lot F).

In the case of the halopheniramine maleates, all samples were dried at 105°C, contrary to the compendial procedures. This deviation was justified for 3 reasons. First, the compendial procedures differ in specifying drying at 105°C for 3 h for the racemates, and 65°C for 4 h for the dextrorotatory isomers; similar treatment of both isomers seemed to be desirable. Second, at least one of each pair of isomers has been characterized by a crystal structure determination; thus decomposition or polymorphic change could be more readily detected. Third, none of the compounds are reported to form hydrates, nor were they observed to be hygroscopic. Because the other drug substances in this study were all dried at 105°C for periods ranging from 1 to 4 h, the higher temperature was chosen.

Powder Data

The various tables report X-ray powder diffraction data in a standardized format (8). (An excerpted version of ref. 8 is available from the JCPDS-International Centre for Diffraction Data, 1601 Park Lane, Swarthmore, PA 19081.) The 2θ values reported are those obtained after the application of the calibration correction. In each case, the *d*-spacings are reported to a number of significant figures corresponding to the precision of the 2θ measurements. Intensities (I/I_1) are reported for each line, relative to the strongest line as 100. Where the data could be successfully indexed, the *hkl* values for any *d*-spacing within ±0.20° (corresponding to the JCPDS limits for an "i" or "indexed" card entry) are reported. Other lines are indicated as "unindexed" and may result from contamination of the sample with another polymorph, solvate, or stereoisomer of the drug, as well as from possible chemical impurities. The indexing calculations were carried out, in part, using the computer program NBS*AIDS80 (9).

The precision of the indexing is stated in terms of the quantitative figure of merit, F_n, as defined by Smith and Snyder (10). Higher values of F_n imply greater agreement between the observed and calculated *d*-spacings. However, F_n is the product of the reciprocal average absolute difference between observed and calculated values of 2θ, taken over all indexed lines, and the fraction of theoretically possible lines which are indexed. To show the relative significance of each of these terms to the combined value, F_n is stated in the following form:

$$F_n\,[\text{average}\,(|\Delta 2\theta|),\ \text{n(possible)}]$$

The "best" indexing will have a large value of F_n, combined with a small average ($|\Delta 2\theta|$) and a value of n(possible) which

Table 5. X-ray powder diffraction data for propranolol hydrochloride, USP

2θ	d(Å)	I/I_1	hkl
8.260	10.70	15	−1,0,1
9.681	9.13	34	101
12.405	7.13	79	110
12.705	6.96	45	200
16.590	5.339	64	−2,0,2
17.050	5.196	45	−2,1,1
18.512	4.789	15	211
19.153	4.630	41	3,0,−1
19.473	4.554	63	202
19.754	4.490	37	−2,1,2
21.115	4.204	48	301
21.956	4.045	28	310
22.196	4.001	18	212
23.017	3.861	5	−1,2,1
23.558	3.773	24	121
23.999	3.705	7	−3,1,2
25.000	3.559	100	220
25.280	3.520	10	−2,2,1
25.601	3.477	10	−1,0,4
26.302	3.385	12	221
27.023	3.297	29	312
27.803	3.206	6	−1,1,4
29.406	3.035	18	411
30.487	2 930	3	−3,2,2
31.368	2 849	12	313
31.668	2 823	5	−4,1,3
31.969	2 797	5	−1,0,5
32.389	2 762	1	030
32.950	2 716	4	322; −2,0,5
33.451	2.677	9	−1,3,1
33.872	2.644	9	131
34.932	2.566	7	230
36.814	2.439	2	−3,1,5
37.897	2.372	8	330
38.977	2.309	3	600
40.538	2.223	3	610; 016
41.120	2.193	5	324
42.462	2.1270	2	−5,0,5; −3,1,6
46.366	1.9566	6	530; 126
47.126	1.9268	2	−3,0,7
48.508	1.8751	2	−5,3,3; −6,1,5
48.950	1.8592	1	341
50.052	1.9208	2	505

is not much greater than n. However, the reality of the way in which most organic compounds crystallize leads to a large number of *d*-spacings which could be observed, but are not because of low scattering. Organic compounds can, therefore, be considered as having been successfully indexed if F_n is greater than 8, and probably successfully indexed if the value is greater than 5, in contrast with higher values usually found for the more symmetrical and more highly scattering inorganic compounds.

The X-ray powder diffraction data for furosemide, USP, are given in Table 1. The melting point of the Reference Standard was 211.0–212.5°C [lit. 206°C (3)].

The X-ray powder diffraction data for hydrochlorothiazide, USP, are given in Table 2. Because of the reported variability of the melting point of this drug substance [see Deppeler (11), and references therein], no attempt was made to determine this property.

X-ray powder diffraction data for naproxen, USP, and naproxen sodium, USP, are reported in Tables 3 and 4, respectively. The melting point of USP Naproxen Reference Standard was 154.4–156.2°C [lit. 155.3°C (3)], and that of USP Naproxen Sodium Reference Standard was 252.6–255.3°C [lit. 244–246°C (12)].

The X-ray powder diffraction data for propranolol hydrochloride, USP, are given in Table 5. The melting point of the Reference Standard was 162.7–164.0°C [lit. 163–164°C (3)].

The X-ray powder diffraction data for brompheniramine maleate, USP, and dexbrompheniramine maleate, USP, are given in Tables 6 and 7, respectively. The melting point of the Reference Standard was 132.0–133.4°C [lit. 132–134°C (3)] for the racemate and 105.0–107.1°C [lit. 106–108°C (13)] for the dextrorotatory isomer.

The X-ray powder diffraction data for chlorpheniramine maleate, USP, and dexchlorpheniramine maleate, USP, are given in Tables 8 and 9, respectively. The *Powder Diffraction File* (1, card 29-1614) includes data for only one form, with no specification of stereochemical purity. Comparison of the *Powder Diffraction File* data [derived from the work of Folen (14)] and the more recently published data of Eckhart and McCorkle (15) with Tables 8 and 9 identifies the material for which data are published as the racemate. The melting point of the Reference Standard was 130.7–132.5°C [lit. 130–135°C (3)] for the racemate and 110.6–112.6°C [lit. 113–115°C (3)] for the dextrorotatory isomer.

The powder diffraction data in Tables 1–7 and Table 9 have been accepted for inclusion in the *Powder Diffraction File* as follows: Furosemide (32–1685); Hydrochlorothiazide (33–1698); Naproxen (34–1751); Naproxen Sodium (34–1750); (±)-Propranolol Hydrochloride (32–1870); (+)-Chlorpheniramine Maleate (32–1576); (+)-Brompheniramine Maleate (33–1554); (±)-Brompheniramine Maleate (33–1555).

Discussion

Furosemide

The diffraction patterns of all commercial samples of furosemide examined were identical with that of USP Furosemide Reference Standard. The melting point of furosemide, recrystallized from ethanol, is reported to be 206°C (3). USP XX does not include the solvent of recrystallization or the melting point range among the specifications in the compendial monograph. However, the experimental data, including melting point determinations for the commercial samples, are self-consistent. The disagreement between the reported and the experimental values may be due to a dependence of the melting point on the heating rate.

The width at half-maximum was 0.12° for the 010 reflection. As distributed, USP Furosemide Reference Standard appears to be slightly hygroscopic. For dried, sieved samples, the relative intensities were reproducible to within approximately ±5% of the intensity of the strongest line. The material was also examined without drying, and relative intensities were substantially less reproducible. In particular, the second strongest line in Table 1 (d = 3.587 Å, I/I_1 = 75) often showed a relative intensity of less than 40. The diffraction patterns of the undried samples also showed occasional weak lines which were not reproducible between successive samples, and which are not reported in Table 1. In no case did any such line have a relative intensity exceeding 15.

The *hkl* indices assigned to the data in Table 1 are based on a triclinic unit cell with dimensions: a = 10.467, b = 15.725, c = 9.584 Å, α = 107.27°, β = 115.04°, γ = 93.47°. These are reduced cell dimensions corresponding to the unit cell reported in the single crystal structure determination of furosemide (16). The published cell data may be transformed to the reduced cell data by the matrix 001/100/0,1,−1. For the indexed lines below 25.2°, the quantitative figure of merit is F_{20} = 11.78 (0.0270, 63). The higher angle lines were excluded from the figure of merit because of the "easy matching" characteristic of triclinic unit cells. All of the indices reported in Table 1 yield a difference of less than 0.09° between the observed and calculated values of 2θ. In only 4 cases (001; 1, −3,1; −2,1,2; −2,−3,2) does the difference exceed 0.05°.

Table 6. X-ray powder diffraction data for brompheniramine maleate, USP

2θ	d(Å)	I/I_1	hkl	2θ	d(Å)	I/I_1	hkl
9.101	9.71	34	002	30.387	2.939	3	222
9.281	9.52	20	011	30.647	2.915	6	−2,3,2
10.102	8.75	28	−1,0,2	31.107	2.873	5	−2,3,3
12.164	7.27	36	−1,1,1	32.129	2.783	14	133; −2,3,4
13.025	6.79	36	−1,1,2	32.449	2.757	2	
15.168	5.836	10	111	33.010	2.711	1	
15.328	5.776	12	−1,1,3	33.571	2.667	13	
16.309	5.430	8	102; 020	34.111	2.626	9	
16.550	5.352	4	−1,0,4	34.572	2.592	11	
16.930	5.232	13	021	35.233	2.545	1	
18.292	4.846	2	004	35.893	2.500	1	
18.692	4.743	12	−1,2,1; 022	36.374	2.468	4	
19.213	4.616	57	120; −1,2,2	37.074	2.423	5	
19.473	4.554	6	unindexed	37.555	2.393	6	
20.014	4.433	52	200; 014	37.976	2.367	5	
20.314	4.368	100	−2,1,1; −2,1,3	38.377	2.344	5	
20.895	4.248	43	−1,2,3	39.017	2.307	6	
21.335	4.161	43	023	41.040	2.197	2	
21.916	4.052	23	113; −2,1,4	41.620	2.1681	5	
22.157	4.009	17	−1,1,5	41.921	2.1532	5	
23.338	3.808	3	−1,2,4	42.641	2.1185	2	
23.578	3.770	3	unindexed	43.463	2.0803	1	
24.159	3.681	32	unindexed	44.565	2.0314	3	
24.579	3.619	38	024	44.985	2.0134	2	
24.960	3.564	58	−2,2,3; −1,0,6	45.765	1.9809	4	
25.340	3.512	1	202	46.067	1.9686	3	
25.721	3.461	22	114	46.866	1.9369	6	
25.901	3.437	15	220	47.768	1.9024	3	
26.162	3.403	58	123; −2,0,6	47.947	1.8957	3	
26.382	3.375	45	032; −1,2,5	49.569	1.8374	4	
27.583	3.231	8	−3,0,4	50.611	1.8020	2	
27.823	3.204	17	221	51.272	1.7803	3	
28.184	3.163	10	033	52.394	1.7448	1	
28.365	3.144	10	−3,1,3	53.335	1.7162	2	
29.205	3.055	4	unindexed	54.417	1.6846	2	
30.147	2.962	9	213	56.960	1.6153	2	

A second crystal structure of furosemide has been reported (17). Cell dimensions for this form are reported to be: a = 5.251, b = 8.771, c = 15.038 Å, α = 101.77°, β = 89.05°, γ = 97.57°. The fact that the indexing to this unit cell was somewhat less satisfactory, as shown by the F_n values, in combination with the observation of Lamotte et al. (16) that the 2 cells have the b^*c^* net in common, provides confirmation that the latter cell is a subcell of the former, and not an example of polymorphism. The latter unit cell may be transformed to the reduced cell reported above by the matrix 200/011/−1,−1,0.

Hydrochlorothiazide

The *hkl* indices assigned to the data for hydrochlorothiazide (Table 2) are based on a monoclinic unit cell (18): a = 7.419, b = 8.521, c = 10.003 Å, β = 111.72°. All diffraction lines were indexed satisfactorily. In no case did the difference between the observed and calculated values of 2θ exceed 0.105°. The accuracy of the indexing is given by F_{30} = 14.18 (0.302, 70). No comparisons with commercial samples were made for hydrochlorothiazide. Deppeler (11) has reported that measurements on industrial production lots agree very well with a powder diffraction pattern calculated from the single crystal structural data. The data reported in Table 2 are in acceptable agreement with the more qualitative data reported by Deppeler.

The indexing of the data revealed that the unit cell for hydrochlorothiazide has a metrically orthorhombic A-centered supercell: a = 8.521, b = 18.586, c = 7.419 Å. A satisfactory, though slightly less precise, indexing [F_{30} = 11.55 (0.309, 84)] was obtained for this cell. The existence of such a supercell is suggestive, although not conclusively so, of a possible misassignment of the space group.

Naproxen and Naproxen Sodium

Because no single crystal cell data have been reported for either naproxen or naproxen sodium, indexing is not reported in Tables 3 and 4. Attempts to index the data by using the method of Visser (19) were unsuccessful. (The computer program described in ref. 19 was obtained from G. G. Johnson, Jr, Materials Research Laboratory, Pennsylvania State University, University Park, PA 16802.) The discrepancy between the experimental and literature melting points could not be explained. Differential scanning calorimetry curves show no evidence of phase transitions.

Propranolol Hydrochloride

The *hkl* indices assigned to the data for propranolol hydrochloride (Table 5) are based on a monoclinic unit cell (20): a = 14.017, b = 8.285, c = 14.005 Å, β = 98.76°. These are the reduced cell dimensions corresponding to the unit cell reported in the single crystal structure determination of propranolol hydrochloride. For the indexed lines below 30°, the quantitative figure of merit is F_{23} = 6.71 (0.0434, 79). It is noted that the near-identity of the experimental values for a and c leads to an approximate degeneracy of the *hkl* and *lkh* d-spacings. The slight broadening of most of the diffraction peaks is an apparent consequence of this geometrical fact. For example, the width at half-maximum for *hkl* = 220 is 0.20° at a 2θ value of 25°. Such broadening leads to a slightly increased uncertainty or random error in the measurement of the 2θ values for each maximum. The 2θ difference between the *hkl* and *lkk* powder lines, which is less than 0.015° for 2θ values below 50°, is an additional systematic error to be added to the random error for the 2θ values. This additional contribution to the denominator of F_n (through the $|\Delta 2\theta|$ term) yields a value which is somewhat lower than might be expected,

Table 7. X-ray powder diffraction data for dexbrompheniramine maleate, USP

2θ	d(Å)	I/I_1	2θ	d(Å)	I/I_1
3.133	28.2	17	23.077	3.851	5
4.755	18.57	11	23.458	3.789	5
6.317	13.98	33	24.219	3.672	39
6.537	13.51	48	24.459	3.636	19
9.121	9.69	17	24.840	3.581	100
9.301	9.50	17	25.280	3.520	13
9.521	9.28	6	25.821	3.447	18
9.962	8.87	11	26.302	3.385	48
10.542	8.38	10	27.223	3.273	27
11.063	7.99	5	27.643	3.224	38
12.244	7.22	9	28.365	3.144	13
12.705	6.96	8	28.904	3.086	29
12.925	6.84	37	29.525	3.023	2
13.166	6.72	8	30.367	2.941	7
13.506	6.550	16	30.907	2.891	2
14.387	6.151	7	31.888	2.804	15
15.148	5.844	3	32.869	2.722	8
15.829	5.594	50	33.190	2.697	8
16.209	5.463	29	33.550	2.669	8
16.410	5.397	55	34.412	2.604	6
16.790	5.276	16	35.233	2.545	2
17.311	5.118	3	35.813	2.505	3
18.011	4.921	20	36.213	2.478	1
18.212	4.867	35	36.935	2.432	3
18.712	4.738	14	37.335	2.406	3
19.093	4.644	30	38.216	2.353	6
19.654	4.513	80	39.378	2.286	4
19.994	4.437	40	40.079	2.248	4
20.134	4.406	42	40.340	2.234	4
20.354	4.359	28	41.321	2.183	3
20.594	4.309	18	41.880	2.1552	3
21.195	4.188	67	42.702	2.1156	1
21.636	4.104	5	43.262	2.0895	1
22.317	3.980	9	43.642	2.0722	2
22.757	3.904	35	44.344	2.0410	2

Table 8. X-ray powder diffraction data for chlorpheniramine maleate, USP

2θ	d(Å)	I/I_1
9.321	9.48	7
12.244	7.22	13
12.505	7.07	3
12.945	6.83	30
15.328	5.776	2
16.370	5.410	6
16.610	5.333	2
16.990	5.214	6
18.572	4.773	10
18.732	4.733	7
19.213	4.616	100
20.174	4.398	64
20.795	4.268	14
21.186	4.190	27
21.776	4.078	10
22.276	3.987	3
24.139	3.684	36
24.639	3.610	13
24.980	3.562	18
25.681	3.466	18
26.162	3.403	23
27.884	3.197	6
28.123	3.170	6
28.344	3.146	1
29.906	2.985	5
30.687	2.911	3
32.148	2.782	7
33.631	2.663	5
34.733	2.581	12
35.412	2.533	1
37.495	2.397	3
38.176	2.355	2
41.781	2.1601	3
44.484	2.0349	1
46.766	1.9408	1
48.210	1.8860	1
49.529	1.8388	1

although still indicative of a correct indexing. For clarity, values for only one index of such a pair are shown in Table 5.

The diffraction pattern of a commercial sample was identical with that of USP Propranolol Hydrochloride Reference Standard. Dried samples of propranolol hydrochloride showed values of I/I_1 which were much more reproducible than those from samples which were examined without drying. As obtained, the material is a very fine white powder which appears to be slightly hygroscopic. The resulting tendency to form lumps may well account for the variability in diffracted intensity. Therefore, drying the sample before examination is essential.

An additional fact of significance to the identification of propranolol hydrochloride by compendial methods is that a test for specific rotation is required, with limits stated as "between −1.0° and +1.0° . . ." (2). However, the crystallographic space group of propranolol hydrochloride is $P2_1/n$. This space group is centrosymmetric, and must, by symmetry, contain equal numbers of left- and right-handed molecules. This material, therefore, occurs as a "racemic compound" rather than a "racemic mixture," according to the definitions given by Eliel (21). Comparison of an X-ray powder diffraction pattern with the data in Table 5 or with the *Powder Diffraction File* card yields essentially the same information as the specific rotation test. The reported specific rotation of (+)-propranolol hydrochloride is +22.2° (*c*, 0.99) (22). As in USP XX, the solvent is unspecified, implying water. The stated limits to the specific rotation test would not be exceeded until the sample contained nearly 5% of the dextrorotatory isomer, a level of contamination which probably would be detected by X-ray examination of the bulk drug.

The stereochemically pure dextrorotatory isomer of propranolol hydrochloride, dexpropranolol hydrochloride (USAN) is not, at present, a drug which has been approved for marketing in the United States. Therefore, its X-ray powder diffraction data are not included here. The crystal structure of dexpropranolol hydrochloride has been reported (23), and thus lines in the powder pattern of propranolol hydrochloride which do not match the published pattern can easily be tested to determine if they correspond to possible *d*-spacings of dexpropranolol hydrochloride.

Brompheniramine Maleate

The powder diffraction data reported for brompheniramine maleate (Table 6) have been indexed on a monoclinic unit cell: a = 9.863, b = 10.836, c = 21.494 Å, β = 115.83°. A single crystal structure determination has been reported for the racemate, facilitating the indexing (24). The quantitative figure of merit is F_{36} = 9.014 (0.0444, 100). The higher lines were not indexed because of the decreasing resolution of the pattern. All indexed lines yield diffraction angles which are less than 0.15° from the observed value. Four lines, including one of intensity substantially above background, could not be satisfactorily indexed, suggesting that the sample may contain a second phase. In contrast with chlorpheniramine maleate (see below), these lines are insufficient to support a hypothesis that the second phase is the dextrorotatory isomer, because several of the stronger lines are not observed. However, the racemic modification of brompheniramine maleate is clearly in the form of a racemic compound.

Chlorpheniramine Maleate

The data for dexchlorpheniramine maleate (Table 9) have been indexed on a monoclinic unit cell: a = 5.7669, b = 20.338, c = 9.1347 Å, β = 103.73° (25). The quantitative figure of merit is F_{18} = 7.466 (0.0578, 44). All indexed lines yield diffraction angles which are less than 0.17° from the

Table 9. X-ray powder diffraction data for dexchlorpheniramine maleate, USP

2θ	d(Å)	I/I_1	hkl
8.560	10.32	4	020
10.863	8.14	15	011
16.309	5.430	16	110
16.510	5.365	6	031; −1,0,1
18.572	4.773	4	−1,2,1
19.233	4.611	2	unindexed
19.994	4.437	39	002
20.434	4.342	100	012
20.995	4.228	2	111
21.836	4.067	5	022
22.337	3.977	8	−1,0,2
22.737	3.908	1	−1,1,2
23.518	3.780	6	140
23.899	3.720	10	032
24.480	3.633	3	131
26.562	3.353	4	042
27.023	3.297	2	150
27.503	3.240	2	−1,5,1
30.547	2.924	18	013
31.348	2.851	3	−2,1,1
33.030	2.710	2	033
35.013	2.561	1	043
39.058	2.304	1	2,5,0; 1,6,2

observed value. The lines at the highest angles were excluded from the calculation of F_n because the combined factors of low intensity and broadening made the exact angular position uncertain. A small shoulder was observed on the 012 peak which is not reported in Table 9, but which might be accounted for by either 130 or 101 (2θ of 20.55° and 20.64°, respectively). The single unindexed line in Table 9 corresponds to the strongest line from Table 8, suggesting that the sample may contain a small amount of the racemate as a second phase. The observed differences in the diffraction patterns of the racemate and the dextrorotatory compound are consistent with, but are not absolute proof of, the existence of the racemate as a racemic compound.

Conclusions

The data reported are sufficient for the identification of each of the listed drug substances as the pure bulk drug. Identification of any of these drugs in combination dosage forms may be possible by reference to the *Powder Diffraction File* (1) when excipients are lacking or do not overlap with the principal *d*-spacings of the drug substance. The data for propranolol hydrochloride and brompheniramine maleate, having been indexed by reference to unit cells which have also been shown to be centrosymmetric, have shown these drug substances to exist as racemic compounds. In the case of chlorpheniramine maleate, a similar conclusion is supported by the differences between the data for the racemate and that for the pure stereoisomer.

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Identification of a Propantheline Analog in Propantheline Bromide Tablets

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A major impurity in a sample of propantheline bromide tablets has been identified as 9-hydroxypropantheline on the basis of the proton magnetic resonance (PMR) and mass spectra (MS). This identification was confirmed by methanolysis of the tablet extract, which yielded a mixture of methyl xanthanoate and methyl 9-hydroxyxanthanoate. A liquid chromatographic (LC) procedure is described which will permit quantitation of 9-hydroxypropantheline bromide in the presence of propantheline bromide, xanthone, and xanthanoic acid.

Propantheline bromide (I), or *N,N*-diisopropyl-*n*-methyl-2-(xanthen-9-ylcarbonyloxy)ethylammonium bromide, is an anticholinergic agent used for treatment of peptic ulcer and renal colic. Synthesis of propantheline bromide was reported by Cusic and Robinson (1); further synthetic work was conducted by Buckley et al. (2).

The present pharmacopeial methods for the assay of propantheline bromide tablets are of limited specificity; both the *British Pharmacopoeia* (3) (BP) and the *U.S. Pharmacopeia* (4) (USP) monographs specify a nonaqueous titration procedure. The BP monograph for tablets includes a test for xanthanoic acid; the monograph for the substance includes a general test for related substances. The USP monograph for propantheline bromide substance contains tests for xanthanoic acid and xanthone (5) and assays for bromide and propantheline bromide content. A limit for xanthanoic acid is also included in the USP tablet monograph. The USP Convention (6) has proposed a liquid chromatographic (LC) method for determination of the dissolution of propantheline tablets. The proposed procedure uses a reverse phase column and a mixture of acetonitrile and pH 3.5 acetate buffer as the mobile phase.

An official sample of propantheline bromide tablets was analyzed, using the LC method described later in this paper, and determined to contain only 60% of the stated content of propantheline bromide. The product, which complied with the requirements of the relevant BP 1980 monograph, contained a mixture of propantheline bromide and approximately 40% of a closely related impurity. The work described in this paper details the procedures used to isolate and identify this impurity.

Experimental

Reagents and Apparatus

(a) *Chemicals.*—Propantheline bromide reference substance (Protea Pharmaceuticals Pty. Ltd, NSW, Australia); xanthene-9-carboxylic acid (Aldrich Chemical Co., Madison, WI); xanthydrol (practical grade) (Eastman Organic Chemicals, Rochester, NY); chromium trioxide (Ajax Chemicals Ltd, Sydney, Australia); absolute ethanol (Merck, Darmstadt, GFR); acetonitrile and methanol, both LC grade (Waters Associates, Bedford, MA); formic acid and NaOH, reagent grade (M.B., Dagenham, UK); H_2SO_4, glacial acetic acid, and anhydrous Na_2SO_4 (BDH Chemicals, Poole, UK).

(b) *Liquid chromatograph.*—Varian 8500 liquid chromatograph with Chromatronix Model 230 fixed wavelength detector operating at 254 nm. Waters Associates μBondapak C_{18} column (30 cm × 3.9 mm). Altex 20 μL loop injector.

(c) *Gas chromatograph/mass spectrometer.*—DuPont Model DP 102 gas chromatograph/mass spectrophotometer fitted with glass column packed with 3.0% SP2100 on 80–100 mesh Supelco support; temperature-programmed over the range 100–280°C for 25 min.

(d) *Mass spectrometers.*—AEI Model MS902 and VG Micromass Model VG 70705, used for probe samples.

(e) *Nuclear magnetic resonance spectrophotometer.*—Varian Model CFT 20 operated in proton mode. All samples dissolved in deuterochloroform.

(f) *Infrared spectrophotometer.*—All IR spectra were obtained using ATR technique on Perkin Elmer Model 457 IR spectrophotometer.

Preparation of Standards

(a) *Xanthone.*—Six mL glacial acetic acid was added to a solution of 3.8 g (0.038 mol) chromium trioxide in 6 mL water. Six g (0.026 mol) xanthydrol was dissolved in 30 mL glacial acetic acid with gentle warming. The magnetically stirred xanthydrol solution was chilled in an ice bath. The chromium trioxide solution was cautiously added to the xanthydrol solution at a rate which maintained the temperature of the solution at ca 10°C. The suspension was stirred for 30 additional min and then poured into 100 mL iced water. The mixture was stirred for several minutes and then filtered under vacuum. The residue was washed with 50 mL 0.5M H_2SO_4 and then with water. The residue was recrystallized from absolute ethanol and dried under vacuum over P_2O_5. Yield: 3.7 g (75%); mp 172°C (lit. 170–174°C); MW 196; anal. calculated: C 79.59, H 4.08, O 16.33; found: C 78.15, H 4.09, O 17.53.

(b) *Methyl xanthanoate.*—Three g (0.013 mol) xanthanoic acid was dissolved in 50 mL methanol. Concentrated H_2SO_4 (1 g) was added. The solution was refluxed 16 h under a double surface condenser, diluted with a solution of 2.5 g $NaHCO_3$ in 100 mL water, and extracted with 70 and 50 mL portions of ether. The combined ether extracts were dried over Na_2SO_4 and evaporated. The residue was recrystallized from methanol. The crystals were filtered, washed with a small quantity of methanol, and dried under vacuum over P_2O_5. Yield: 2.28 g (71%); mp 83°C; MW 240; anal. calculated: C 75.00, H 5.00, O 20.00; found: C 75.21, H 5.16, O 20.44.

(c) *Propantheline formate.*—Forty mg propantheline bromide was dissolved in 800 μL chloroform, and 100 μL solution was applied as a streak to each of 6 methanol-washed, pre-coated Merck silica gel 60 F_{254} plates (20 cm × 20 cm). The plates were developed with a mixture of acetonitrile and formic acid (100 + 4). The zone on the plate containing propantheline was cut out with scissors and extracted with developing solvent. The extract was evaporated under vacuum.

Methanolysis Experiments

(a) *Propantheline bromide.*—Propantheline bromide (200 mg) was dissolved in methanol (50 mL) and refluxed 1 h. The solvent was evaporated under vacuum and the residue was dissolved with shaking in a mixture of ether and water. The ether phase was separated, dried over Na_2SO_4, and evaporated. The residue was dissolved in ether (ca 10 mL), and the solution was filtered and evaporated. The IR and nuclear magnetic resonance (NMR) spectra of the residue were identical with those determined for methyl xanthanoate.

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Table 1. Mass spectra of propantheline bromide and analogs

Compound	Mass/charge (daltons)[a]									
Methyl xanthanoate	240	(3.9)	182	(14.2)	181	(100)	152	(7.8)	—	—
Methyl 9-hydroxyanthanoate	256	(0.4)	240	(0.25)	329	(0.25)	225	(0.7)	—	—
	211	(3)	198	(15)	197	(100)	181	(9.3)	168	(17)
	152	(3)	139	(13)						
Propantheline bromide	353	(1.3)	339	(2.0)	338	(8.9)	326	(2.0)	325	(8.0)
	311	(8.1)	310	(43.8)	182	(25.0)	181	(100)	152	(23.2)
9-Hydroxypropantheline formate	369	(0.02)	368	(0.02)	354	(0.1)	341	(0.6)	326	(2.4)
	325	(0.2)	310	(0.4)	304	(0.9)	270	(0.8)	235	(1.9)
	225	(1.5)	197	(100)	181	(40)	168	(3.6)	—	—
	152	(2.8)	139	(3.8)	—	—	—	—	—	—

[a]The ion abundance is given as a percentage of the base peak in parentheses after each ion.

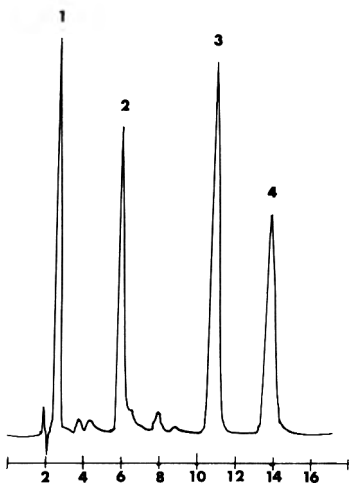


Figure 1. Chromatogram of spiked defective tablets; retention time in minutes. Peak identity: (1) 9-hydroxypropantheline; (2) propantheline; (3) xanthanoic acid; (4) xanthone.

(b) *Tablet extract.*—Ten tablets were triturated twice with ether, and the ether solution was discarded. The solid residue was triturated twice with 25 mL portions of methanol, and the supernate was filtered, refluxed 1 h, and then reduced in volume under vacuum. The precipitated material was removed by filtration, and the filtrate was evaporated to dryness and dissolved with shaking in a mixture of ether (25 mL) and water (25 mL). The aqueous phase was separated and extracted with 25 mL ether and the combined extract was reduced in volume.

The presence of xanthone, methyl xanthanoate, and methyl 9-hydroxyxanthanoate in the ether extract was confirmed by GC/MS; retention times were xanthone 12.4 min, methyl xanthanoate 13.2 min, methyl 9-hydroxyxanthanoate 14.1 min.

Preparative TLC (silica gel) of the ether extract solution yielded methyl xanthanoate and a compound characterized as methyl 9-hydroxyxanthanoate: mp 113–115°C; anal. calculated: C 70.31, H 4.69, O 25.00; found: C 70.21, H 4.99, O 25.31.

Thin Layer Chromatography

(a) *Analytical thin layer chromatography.*—The tablets were extracted by triturating twice with ether to obtain the less polar compounds and then twice with chloroform. Samples of the ether and chloroform extracts, xanthone, xanthanoic acid, and propantheline bromide solutions were applied to the plates.

Analytical TLC was performed on Merck pre-coated silica gel F_{254} plates. The plates were developed in a mixture of 1,2-dichloroethane, methanol, and formic acid (80 + 20 + 2).

Plates were visualized by fluorescence quenching under short wavelength UV light (254 nm). Alternatively, the plates were sprayed with 50% H_2SO_4, placed 10 min in a 110°C oven and viewed under long wavelength (360 nm) UV light. When treated in this manner, xanthone fluoresced blue, propantheline and xanthanoic acid fluoresced yellow-green, and 9-hydroxypropantheline fluoresced chocolate brown. R_f values were xanthone 0.73, xanthanoic acid 0.60, propantheline 0.28, and 9-hydroxypropantheline 0.17.

Methyl xanthanoate and methyl 9-hydroxyxanthanoate were separated on silica gel GF_{254} plates (Merck Order No. 7730). The chromatograms were developed with a mixture of 1,2-dichloroethane and methanol (98 + 2). When the chromatograms were visualized by spraying with acid and viewing under long wavelength UV light, methyl 9-hydroxyxanthanoate fluoresced brown and methyl xanthanoate fluoresced yellow-green. R_f values were methyl xanthanoate 0.75, methyl 9-hydroxyxanthanoate 0.50.

(b) *Preparative thin layer chromatography.*—Five tablets were triturated with 25 mL ether, the supernate solution was discarded, and the solid residue was triturated with two 25 mL portions of chloroform. The combined extracts were filtered through a Whatman 540 paper and evaporated to dryness under vacuum. The residue was dissolved in 4 mL chloroform, and 250 mL solution was applied to each of 6 methanol-washed, pre-coated Merck silica gel F_{254} plates (20 cm × 20 cm). The plates were developed in acetonitrile and formic acid (96 + 4). Two major bands were observed under UV (254 nm) light. The lower band was separated from the plate and extracted with developing solvent. The extract was filtered and evaporated under vacuum, yielding 16.6 mg oil which showed only one peak by LC. The retention volume of this peak corresponded to the retention volume obtained for the impurity observed in the tablets.

Assay Procedure

Twenty tablets were accurately weighed and ground to a fine powder. An amount equivalent to ca 50 mg propantheline bromide was weighed into a 100 mL volumetric flask. The powder was extracted with 80 mL 1% acetic acid in methanol solution with vigorous shaking. The solution was then diluted to volume with 1% acetic acid solution. The resulting solution was filtered through Whatman No. 42 paper; 20 μL filtrate was injected onto the column under the following conditions: mobile phase: methanol–0.005M pentane sulfonic acid (55 + 45); flow rate 1.5 mL/min.

Results and Discussion

By using the LC system described above, the impurity, xanthone, and xanthanoic acid were separated from pro-

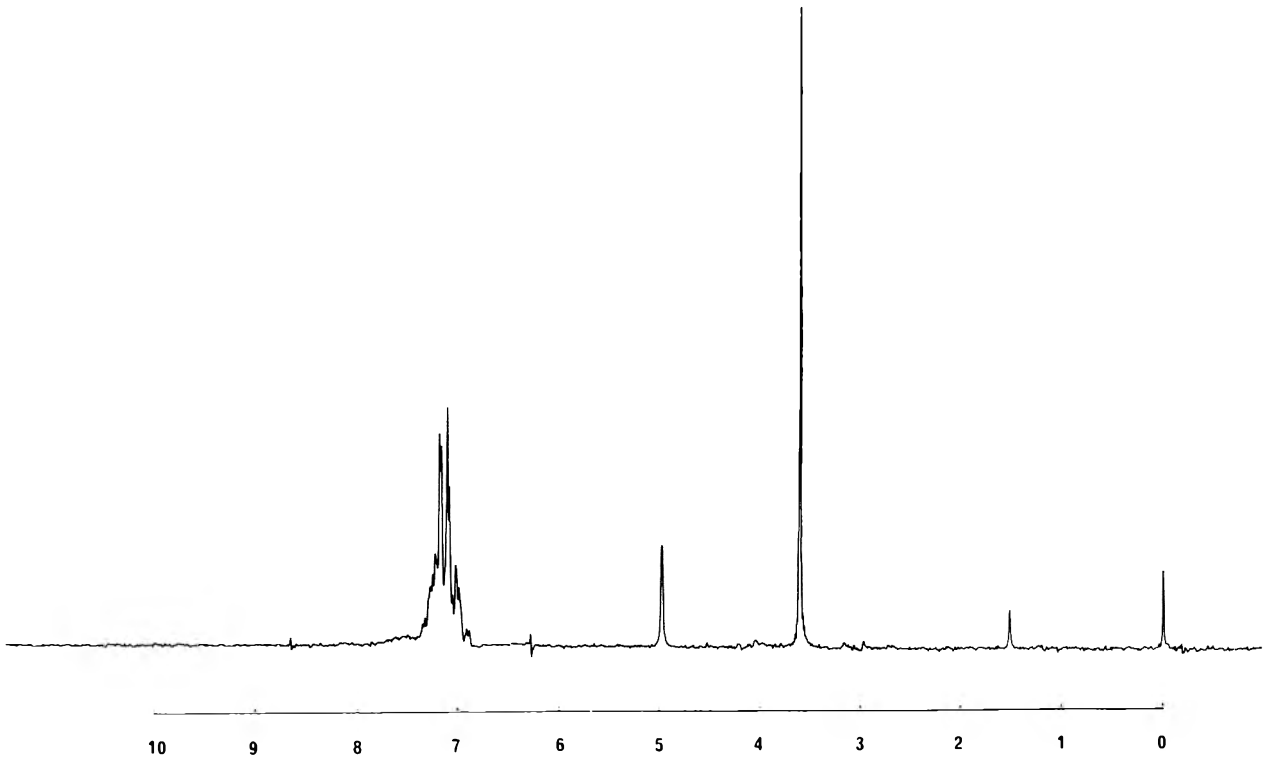


Figure 2. PMR spectrum of methyl xanthanoate; chemical shift: ppm. See Figure 1 for peak identification.

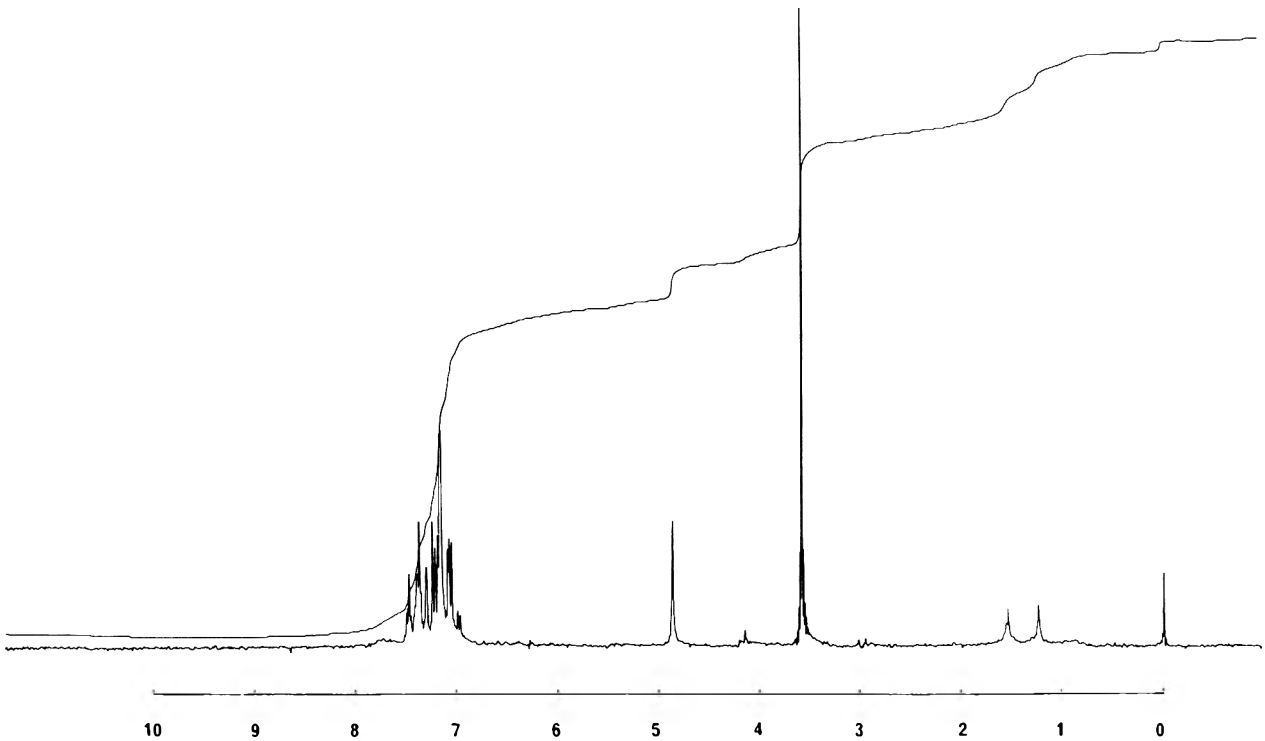


Figure 3. PMR spectrum of methyl 9-hydroxyxanthanoate; chemical shift: ppm. See Figure 1 for peak identification.

pantheline bromide (Figure 1). Elution order of the compounds suggested that the impurity was more polar than propantheline bromide, so a 2-stage extraction procedure for the preparative TLC was devised. This extraction procedure was based on the USP (5) test for xanthone and xanthanoic acid. The preparative TLC system chosen initially was the same as the analytical TLC procedure, and both procedures were based on the related substances test of the BP. This system yielded fractions that were identified as xanthone and xanthanoic acid by IR, proton magnetic resonance (PMR), and mass spectrometry; however, the spectra indicated that the fraction containing the impurity was a mixture of compounds. Analytical TLC of this fraction showed propantheline bromide, the impurity, and one compound which had not previously been observed.

The stability of propantheline bromide under the TLC conditions was studied. It was found that inclusion of methanol in the developing and extraction solvents resulted in transes-

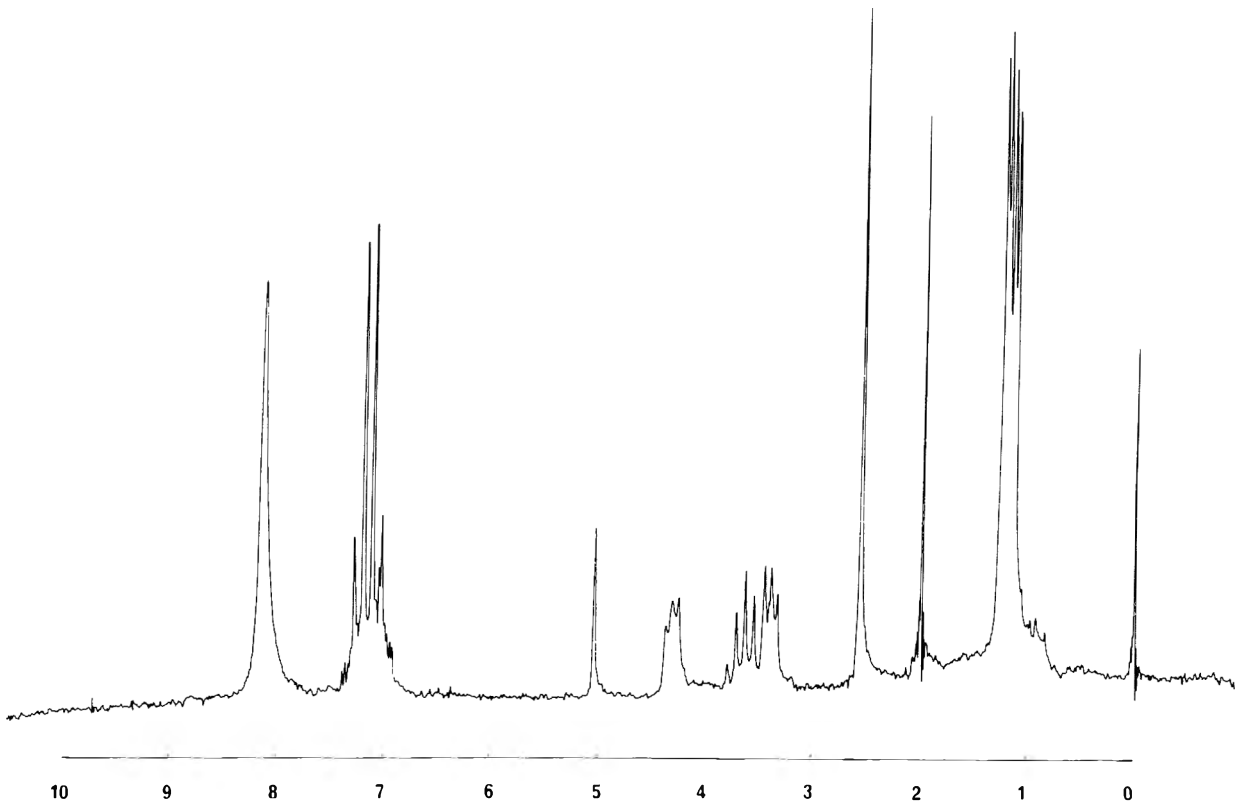


Figure 4. PMR spectrum of propantheline formate; chemical shift: ppm. See Figure 1 for peak identification.

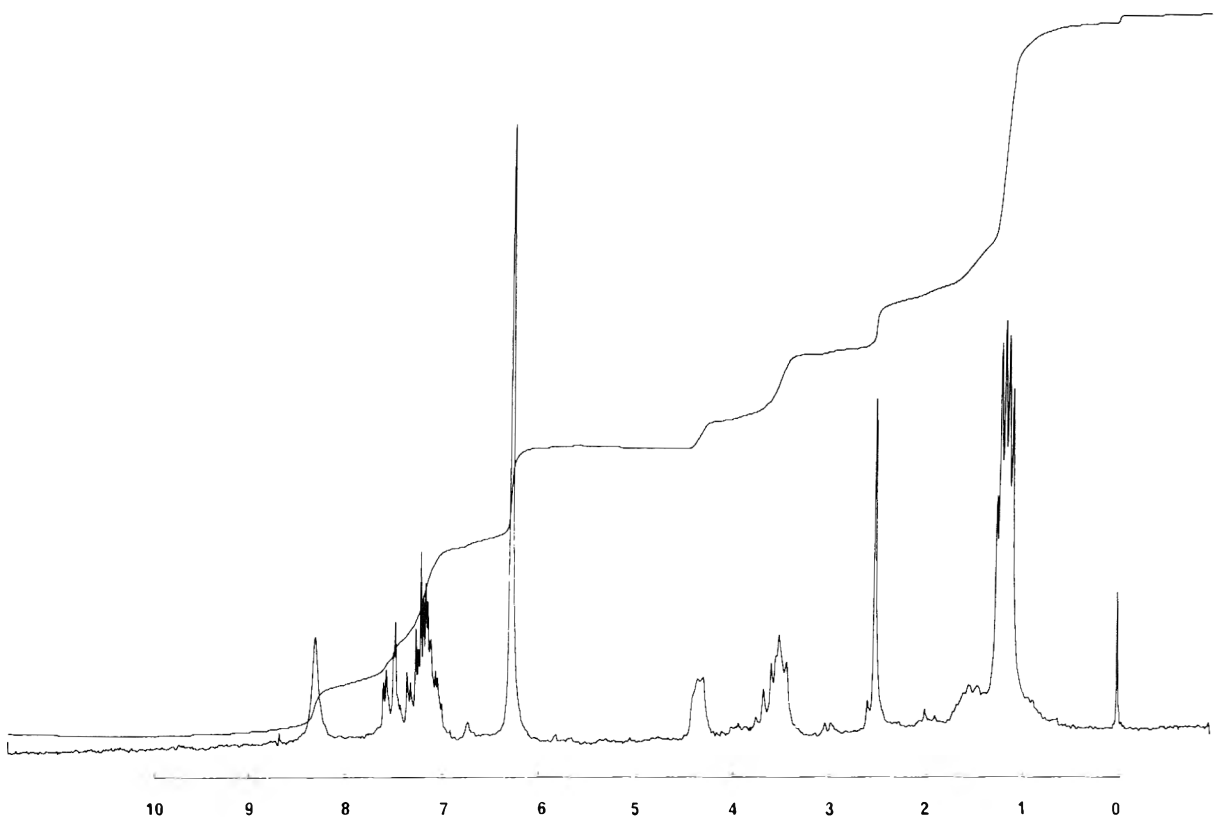


Figure 5. PMR spectrum of 9-hydroxypropantheline formate; chemical shift: ppm. See Figure 1 for peak identification.

terification of propantheline bromide to give methyl xanthanoate. This was confirmed by refluxing propantheline bromide in methanol to give methyl xanthanoate. The susceptibility of propantheline to nucleophilic attack has also been observed by Brown and Scaplehorn (7), who found that propantheline bromide treated with 0.880M ammonia during a basic extraction procedure converted readily to xanthene-9-carboxylic acid amide. The reaction of propantheline bromide with methanol was utilized to prepare the methyl esters which were more readily characterized than the corresponding salts. The mass spectra of the compounds obtained, methyl xanthanoate (III) and methyl 9-hydroxyxanthanoate (IV) (Table

Table 2. PMR spectra of propantheline bromide and analogs

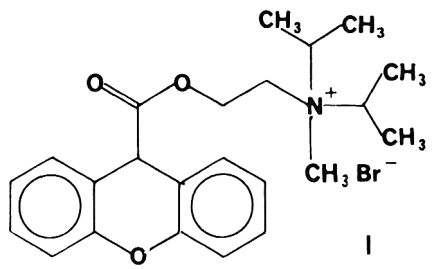
Assignment	Propantheline formate	Propantheline bromide	9-Hydroxypropantheline formate
Methyl protons of isopropyl moiety	δ 1.21 (m) 12 H	δ 1.26 (q) 12 H	δ 1.15 (m) 12 H
Impurity	δ 2.00 (s)	δ 1.61 (s)	
N-Methyl	δ 2.58 (s) 3 H	δ 2.76 (s) 3 H	δ 2.50 (s) 3 H
N-Methylene	δ 3.41 (t) 2 H	δ 3.82 (m) 4 H	δ 3.52 (m) 4 H
Methine	δ 3.70 (q) 2 H	δ 3.82 (m) 4 H	δ 3.52 (m) 4 H
O-Methylene	δ 4.34 (t) 2 H	δ 4.39	δ 4.38 (unresolved multiplet) 2 H
Bridgehead	δ 5.07 (s) 1 H	δ 5.07 (s) 1 H	δ 6.28 (water) 5.5 H
Aromatic	δ 7.20 (m) 8 H	δ 7.28 (m) 8 H	δ 7.34 (m) 8 H
Formate/formic acid	δ 8.20 (s)		δ 8.35 (s)

1) included ions corresponding to the molecular ions (240 and 256) and the loss of the ester moiety (181 and 197, respectively). The mass spectrum of the hydroxy ester includes an ion at 239 which becomes the base peak of the chemical ionization spectrum. This ion may arise through loss of a hydroxyl radical from the molecular ion in the electron impact spectrum or the loss of water from the M + 1 ion in the chemical ionization process.

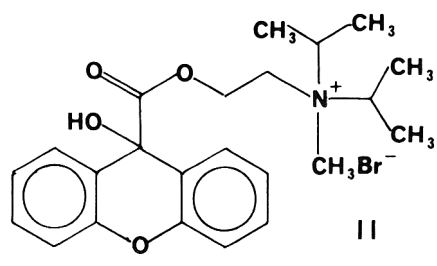
The PMR spectra of methyl xanthanoate and methyl 9-hydroxyxanthanoate are shown in Figures 2 and 3, respectively. The PMR spectrum of the 9-hydroxy ester was similar to the spectrum of methyl xanthanoate with the methyl signal at a virtually identical position in both spectra. However, the aromatic signals were more dispersed for the 9-hydroxy ester. The signal due to the hydroxyl proton had a chemical shift similar to that of the bridgehead hydrogen of methyl xanthanoate; however, after deuterium exchange this signal was removed entirely. The proton at the bridgehead position of methyl xanthanoate did not exchange under the same conditions, confirming the location of the hydroxyl moiety.

The replacement of methanol with acetonitrile in the preparative TLC system enabled the isolation of 9-hydroxypropantheline formate (II) which was identified by PMR and mass spectroscopy. The mass spectra of the salts propantheline bromide and 9-hydroxypropantheline formate did not contain a parent ion because propantheline or 9-hydroxypropantheline were not volatilized. The propantheline bromide spectrum contained peaks resulting from demethylation (353), desisopropylation (325), loss of an isopropyl and a methyl group (310), and loss of the ester moiety (181). The mass spectrum of 9-hydroxypropantheline formate showed the corresponding ions 16 mass units higher at mass/charge 369, 341, 326, and 197, respectively.

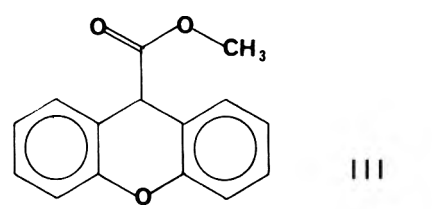
PMR spectra of propantheline formate and 9-hydroxypropantheline formate are shown in Figures 4 and 5, respectively, and the chemical shifts and assignments are shown in Table 2. Propantheline formate was prepared to examine the effect of the counter ion on the PMR spectrum of propantheline as some unexpected differences between the propantheline bromide spectrum and the 9-hydroxypropantheline formate spectrum were observed. Replacement of the formate counter ion with bromide results in a down field shift of all the signals assigned to the side chain and the *N*-methylene and methine signals. The splitting of the isopropyl methyl protons is simplified and more obviously a doublet of doublets. The PMR spectrum of 9-hydroxypropantheline formate differed from the spectrum of propantheline formate because the spectrum did not contain a signal corresponding to the bridgehead proton of propantheline. The aromatic signals were more dispersed than the corresponding propantheline signals. There were minor changes in the chemical shifts of the *N*-methyl and *N*-methylene peaks. As a result, the *N*-methylene and methine signals were not resolved. The isopropyl methyl multiplet also showed increased complexity in its splitting.



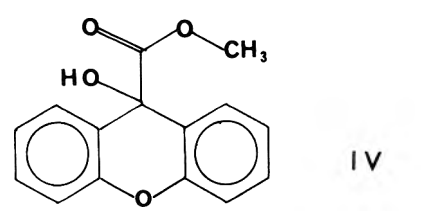
I



II



III



IV

Vose et al.(8) found a phenolic derivative of propantheline bromide as a metabolite of the same drug; however, the authors did not report any data concerning the pharmacology of the compound. During the progress of this work, Ravenscroft reported that tablets from the batch in question displayed a reduced therapeutic effect when compared with other propantheline bromide tablets (P. Ravenscroft (1981)

Princess Alexandria Hospital, Brisbane, Qld, Australia, personal communication). These investigators also found that the batch of tablets contained a considerable amount of an unknown impurity. The 9-hydroxypropantheline bromide in these tablets most probably arises from a carryover in the synthesis of propantheline bromide, and it may be appropriate to set a limit for the content of this compound. The value of this limit will depend on the pharmacological activity of 9-hydroxypropantheline bromide.

The ease of hydrolysis of the ester group suggests that propantheline bromide tablets may exhibit stability problems. A 6-year-old retention sample, which had been assigned a 5-year shelf life by the manufacturer, consisted of a mixture of xanthone and xanthanoic acid. The stability of this product deserves further investigation, and application of the LC method described above should enable measurement of the extent of degradation in propantheline bromide tablets and provide more meaningful stability profiles for these products.

Acknowledgments

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Difference Spectrophotometric Estimation of Santonin

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Santonin gives a characteristic alkaline vs acidic difference spectrum. This was used for its estimation in pharmaceuticals and in the crude drug. Santonin was first extracted and purified through a specific partition procedure; then the difference absorbance was measured either at the maximum, 285 nm, or the minimum, 242 nm. The percentage of santonin can be calculated either by reference to the difference absorbance of a reference santonin sample, treated similarly, or by making use of the determined absorptivity. Measurement at the maximum is advisable, especially when the crude drug is assayed, because natural contaminants may interfere with the difference absorbance at the minimum.

Santonica, the unexpanded flower head of *Artemisia cina*, has long been used as an anthelmintic for roundworms. Its lactonic active principle, santonin, is official in most universal pharmacopeias (1). The crude drug is still official and in current use as folklore medicine in Egypt.

Pharmaceutical preparations of santonin (BPC, 1963) include single-component tablets (BPC, 1958) and tablets of binary mixtures with calomel (BPC, 1954) or phenolphthalein (2). Santonin injection is also official in the Japanese Pharmacopeia (1).

Reported methods for estimating santonin include gravimetric assay (3–5), in which the crystallized active principle is weighed after being isolated and purified through a series of partition purification steps, and sometimes additional chromatographic purification steps. Other published methods are colorimetric estimations (6–9) that depend on a variety of color reactions which are neither specific nor quantitative. Still other methods estimate the lactone volumetrically by isolating it in a relatively pure form, cleaving the lactone ring by standard alkali, and back-titrating the excess alkali (10). The literature also reports polarographic methods (11, 12), infrared methods (13), and liquid chromatographic (LC) methods (2).

Almost all reported methods specify partition purification steps that are based on conversion of the lipophilic lactone to the hydrophilic alkali salt of the corresponding hydroxy-acid by the action of hot fixed alkali. In the present work, santonin was assayed by the difference spectrophotometric technique, which proved to be advantageous over direct spectrophotometric estimation because it eliminated spectral interferences of coexisting contaminants.

Experimental

Apparatus and Reagents

(a) *Spectrophotometer.*—Double beam, self-recording spectrophotometer, capable of measuring absorbance in the range 220–400 nm.

(b) *Reference santonin.*—Santonin D.A.B.-6 (Chimpharm, Bulgaria) was used in all work to measure absorptivities, to compound artificial mixtures, and to enrich the crude drug.

(c) *Santonin solution.*—Dilute reference santonin 1:1000 in methanol.

(d) *Santonica.*—Different commercial samples were used.

(e) *Other reagents.*—Barium hydroxide, saturated solution; 0.1N NaOH; 0.1N HCl.

Recording the Difference Spectra

Record alkaline vs acidic difference spectrum of santonin in the range 220–250 nm as follows: Dilute duplicate aliquots of santonin methanolic stock solution to desired concentration, one with 0.1N HCl (solution A) and the other with 0.1N NaOH (solution B). Record alkaline vs acidic difference spec-

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trum by placing solution A in reference cuvet and solution B in sample cuvet.

The linear relationship between absorbance (at 285 and 242 nm) and concentration was demonstrated by recording the difference spectra for several concentrations of santonin, viz, 1:20 000 to 1:200 000 solutions, and a rectilinear curve was obtained (Figure 1).

Determination of Absorptivity

The difference absorptivity of santonin was measured at the maximum (a_{285} = 9.56) and the minimum (a_{242} = 6.04). However, the absorptivity at both wavelengths decreased about 13.6% from expected values when aliquots of reference santonin solution were subjected to the entire process of extraction, saponification, and partitioning that is necessary to assay the crude drug. Thus, a correction factor (1.13) was introduced into the calculations of santonin content when reference santonin was lacking.

Assay Procedure

Extract 1.0 g powdered drug by heating 15 min on a water bath under a reflux condenser with 20 mL $CHCl_3$. Repeat extraction process twice with further 10 mL portions of $CHCl_3$. Filter each $CHCl_3$ fraction through the same small piece of cotton into a boiling flask. Concentrate combined extracts to ca 5 mL. Add 5 mL saturated $Ba(OH)_2$ solution, evaporate residual $CHCl_3$, then saponify by heating 15 min under a reflux condenser. Let settle, then filter through a small plug of cotton into a 25 mL volumetric flask. Repeat heating, under reflux, for 5 min, with 2 additional 5 mL portions of $Ba(OH)_2$ solution, filtering each fraction through the same filter into the volumetric flask dilute to volume with water.

Transfer 10.0 mL aliquot of solution to small (50–100 mL) separatory funnel, and extract by shaking with 5 mL $CHCl_3$. Transfer $CHCl_3$ layer to another separator and wash with 5 mL water. Combine aqueous solutions, acidify with 10N HCl (1.5 mL), and extract with successive portions of 20, 10, 10, and 10 mL benzene. Wash combined benzene solution with 5 mL water, evaporate benzene solution completely in boiling flask, and dissolve residue in 25.0 mL methanol. Transfer duplicate aliquots, each 5.0 mL, into 25 mL volumetric flasks; dilute one with 0.1N HCl (solution A) and the other with 0.1N NaOH (solution B). Measure difference absorbance (alkaline vs acidic) at 285 nm, by placing solution A in reference cell and solution B in sample cell of spectrophotometer.

Continue as under (A) or (B) below, depending on whether a reference santonin sample is available.

(A) *If reference santonin is not available:* Calculate percentage santonin in sample according to the following formula:

$$\text{Santonin, \%} = A_t/a \times 25/1000 \times 25/5 \times 25/10 \times 1.13 \times 100/\text{g sample} = 3.7A_t/\text{g sample}$$

where A_t = difference absorbance of test solution at 285 nm; a = absorptivity of santonin at 285 nm (9.56); and 1.13 = correction factor for recovery of santonin.

(B) *If reference santonin is available:* Saponify 10 mg santonin by heating 15 min on water bath under a reflux condenser, and dilute to 25 mL with water. Continue as in second paragraph of *Assay Procedure* ("Transfer 10.0 mL aliquot . . ."); then calculate percentage santonin in assayed sample according to the following formula:

$$\text{Santonin, \%} = A_t/A_s \times \text{mg reference santonin}/1000 \times 100/\text{g sample}$$

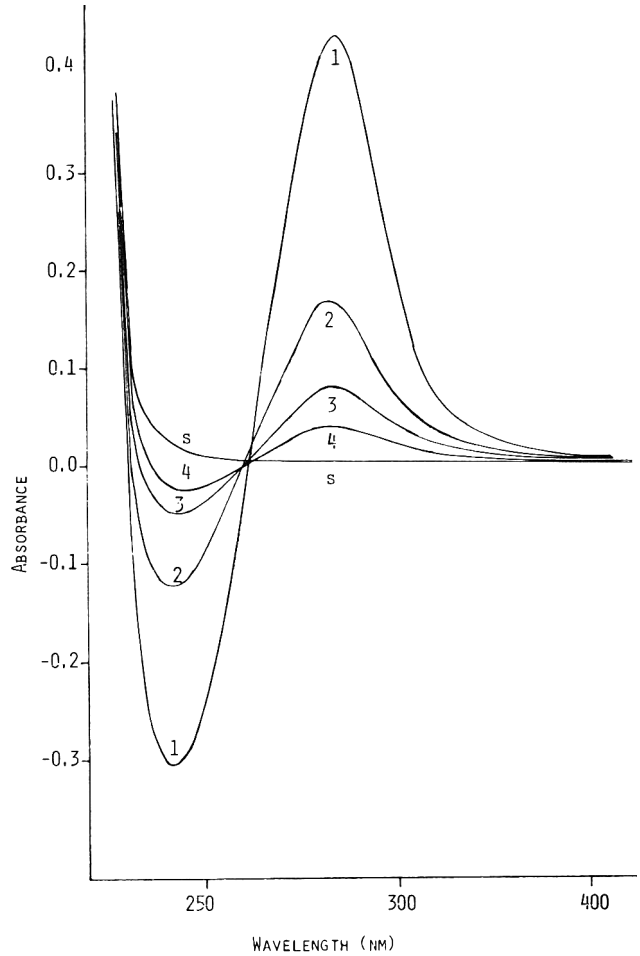

Figure 1. Alkaline vs acidic difference spectra of santonin solutions (1 = 1:20 000; 2 = 1:50 000; 3 = 1:100 000; 4 = 1:200 000, and s = solvent).

where A_t = difference absorbance of test solution at 285 nm; A_s = difference absorbance of reference solution at 285 nm.

Results and Discussion

A study of the conventional ultraviolet spectrum of santonin showed its maximum absorption to be at 240 nm (log ϵ = 4.12). However, attempts to develop a spectrophotometric finish for the assay procedure were hampered by the spectral interferences in the purified lactonic fraction. As expected, partition purification of the cleaved lactonic fraction did not eliminate acids, flavonoids, or other phenolic plant constituents. The presence of these compounds resulted in high absorbance for the purified lactonic fraction of santonica.

We believed that this drug could be a good candidate for assay by difference spectrophotometry, a technique that is recommended when solubility characteristics make separation of spectral contaminants difficult (14).

The extraction procedure was developed using chloroform in the preliminary extraction step, and benzene for extraction of the lactone in the final step. The latter solvent proved to be a good extractant for the lactone but not for the polar spectral contaminants. This was tested by assaying an enriched sample by a modified version in which chloroform was used in the final extraction step; the chloroform extract was slightly colored, and results deviated from the calculated values. Measurement of the difference absorbance of a lactonic fraction, purified in this way would negate the interference of most absorbing contaminants (14).

Table 1. Estimation of santonin in artificial samples, the crude drug, and enriched crude drug by the difference spectrophotometric method

	Santonin content, %		
		Found at	
Sample	Calcd	Max.	Ampl.
Artificial:			
1	10	10.1	10.12
2	5	4.99	5.00
3	2	2.12	2.19
4	1	1.02	1.02
5	0.5	0.47	0.52
Crude drug		2.81[a]	
Enriched crude drug:			Rec., %
1	3.81	3.83	100.5
2	4.81	4.61	95.8
3	5.81	5.51	94.8
4	6.81	6.91	101.5

[a]Relative standard deviation = 1.68 (n = 3); calculated for crude drug.

The alkaline vs acidic difference spectrum of santonin showed a strong maximum at 285 nm (a = 9.56), moderate minimum at 242 nm (a = 6.04), and an isosbestic point at about 260 nm (Figure 1); the latter feature proved to be useful for the detection of spectral impurities in the different purification trials.

The santonin difference spectrum was shown to be consistent and stable for hours. The fact that this spectrum was consistent only for fixed alkali hydroxides suggests a lactone cleavage in the fixed alkali solution. Hence, the difference spectrum is of cleaved vs intact santonin, rather than a pH-induced difference spectrum of either form. This is in accordance with observations by Doyle (T. D. Doyle, Food and Drug Administration, private communication, 1982), who stated that an alkaline solution of hydrolized santonin, when made acid, changes smoothly but slowly over several hours to give a spectrum identical to the acid solution. Therefore, it is evident that the ring opening, and not the ionization, is responsible for the observed difference spectrum.

The difference absorbances at both the maximum (285 nm) and the minimum (242 nm) and, hence, also of the amplitude (242–285 nm), all showed a linear relationship with concentration (Figure 1). Plots of difference absorbance against concentration were also linear.

Results of enriched crude drug assay, however, showed concordance with calculator values only for assays measured at the maximum rather than at the minimum or the amplitude; the latter led to serious deviation. This is understandable from the fact that natural acidic and phenolic contaminants would interfere more with the difference absorbance at the lower wavelength (14, 15).

Santonin is by far the major lactonic principle of the crude drug. However, related minor lactones that are always included in official assays would be eliminated in the present method, to a large extent, by measuring with the difference spectrophotometric technique at the specific maximum of santonin.

The reliability of the method was determined by assaying different artificial and crude drug samples (Table 1). Assaying artificially enriched samples of santonica showed the assay procedure to be of reliable accuracy and reproducibility. Results in Table 1 for the artificial samples of santonin are based on both the difference absorbance at 285 nm and the amplitude at 242–285 nm. Both measurement alternatives proved to be reliable. The results given for the crude and the enriched drug, however, are based on measurement at the maximum only.

Acknowledgment

The authors thank T. D. Doyle, Food and Drug Administration, Washington, DC, for his valuable review comments, and for allowing us to report his finding about the spectrum of hydrolyzed santonin in our discussion section.

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MICROBIOLOGICAL METHODS

Comparison of Helium Leak Test and Vacuum Leak Test Using Canned Foods: Collaborative Study

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Two can leak tests were compared by 7 collaborators. In the helium leak test, pressurized helium is applied to the outside of the container, and a headspace gas sample from the can is then analyzed for the presence of helium. The vacuum test is described in the *Bacteriological Analytical Manual*. Ninety No. 303 cans of creamed-style corn, green beans, carrots, fruit cocktail, and whole-kernel corn were shipped in 3 groups. Two groups of 30 cans had 10 dented flat cans, 5 flat controls (nondented), 10 dented swollen cans, and 5 swollen control cans (nondented). The third group had 10 dented swollen cans and 5 swollen control cans. Of 600 cans analyzed, 37 (6.2%) were deleted from the analysis because results were not available for both tests. One laboratory was constrained by scheduling to analyze 15 of 45 swollen cans. The helium leak test found 12 (13%) positives of 92 nondented swollen cans. One pressurization test yielded 7 of those 12 positives. Of the 400 dented cans sent as possible leakers, the helium test found 267 positives, and the vacuum test found 181. Five of the 7 analysts had significantly ($\alpha = 0.05$) higher percent positive helium results. One analyst found more leakers by the vacuum leak test. Both tests found fewer positives in the swollen dented cans than in the flat dented cans. After exposure to pressurized helium, all cans with greater than 8 psi headspace pressure were positive helium leakers. The method was adopted official first action.

The most frequent source of bacterial contamination of low-acid canned foods is leakage after processing. Although underprocessing does occur, most contamination (spore and non-spore) results from leaks in malformed or dented cans. No official leak detection tests exist (1), but the *Bacteriological Analytical Manual* (BAM) (2) lists 4 microleak procedures (vacuum leak, Mead jar, air pressure, and fluorescein dye tests). The air pressure and fluorescein dye tests are often used to detect the position of a leak in conjunction with seam inspection. Mead jar testing is performed on vacuum- or nonvacuum-packed dried or semidried products. The vacuum leak test is the procedure most frequently used in Food and Drug Administration (FDA) laboratories to screen for leaks in low-acid canned foods.

A recent report (3) indicates that the vacuum leak test may not detect very small leaks. As a result of this and another report (4), a helium leak test was developed. Put et al. (4) reported on a helium procedure in conjunction with mass spectrometry that detected openings as small as 1 μm.

Therefore, a study was designed to compare the helium leak test with the test now most frequently used to screen for microleaks. Because samples could not be prepared that had the same number and size of leak for each collaborator, a comparison of results by both vacuum and helium tests was performed to determine the presence or absence of a leak in typical dented cans (flat and swollen).

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The recommendation of the Associate Referees was approved by the General Referee and by Committee F and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) 67, March issue.

Collaborative Study

A total of 90 No. 303 cans were sent to 7 collaborators in 3 groups. Each collaborator received 15–30 cans in a shipment. The first 2 groups contained 5 cans of flat controls (nondented), 5 swollen controls, 10 dented (presumed leakers) flats, and 10 dented swollen cans. Group 3 consisted of 10 dented swollen and 5 swollen controls. The cans contained creamed-style corn, green beans, carrots, fruit cocktail, and whole-kernel corn.

Collaborators were instructed to pierce cans similar to aseptic procedure **46.021**, because swollen cans would routinely be subjected to microbiological analysis (1, 2) in addition to leak testing. A 1.5 in. hole was cut in the top, and a portion of the contents was discarded. The hole was resealed before the helium test was performed. All cans were placed in a pressurized tank, exposed to helium, then pierced, and tested for helium on a gas chromatograph. In both swollen and flat cans, the end of the can opposite the dent was opened, and the vacuum leak test (2) was performed. The results of the vacuum test were reported as positive or negative. If the percent of helium was ≥ 1, the value was recorded and submitted for analysis.

Bacterial Contamination in Low-Acid Canned Foods
Helium Leak Test
First Action

Principle

He is inert gas with small MW that can be forced through micron size openings and be easily detected by gas chromatgc analysis. After can is pierced aseptically, and sample is taken for microbiological analysis. can is sealed with rubber disk and subjected to He at 45 psi for 30 min. Headspace sample is then taken and analyzed for He.

Apparatus

(a) *Gas chromatograph.*—Instrument capable of sepg He from N, O, H, and CO_2 as described, or equiv., with strip chart recorder, gas partitioner (Fisher Model 1200, Fisher Scientific) with dual thermal conductivity cells and dual in-line columns. Column 1: 6½ ft × ⅛ in. Al packed with 80–100 mesh Columnpak™ PQ. Column 2: 11 ft × 3/16 in. Al packed with 60–80 mesh Molecular Sieve 13 ×.

Operating conditions: column temperature = 75°; attenuation 128; Ar carrier gas inlet pressure 40 psi, flow rate 26 mL/min thru gas partitioner; bridge current 125 mA; column mode 1 & 2; temperature mode, column; injector temp., off.

(b) *Puncturing press (Fig. 46:02).*—Made from drill press for electric hand drill with internal spring reversed to push head down. Metal valve, 3-way (Becton-Dickinson stopcock No. 3161); vac. pressure gage-30 in. Hg-0-60 psi, 2½ in. face (US Gauge, Sellerville, PA 18960). Stainless steel piercer 1½ in. × ½ in. (machined in local machine shop) with No. 2 taper in piercer top, beveled ⅛ in. × 3/16 in. at bottom. ¼ in.

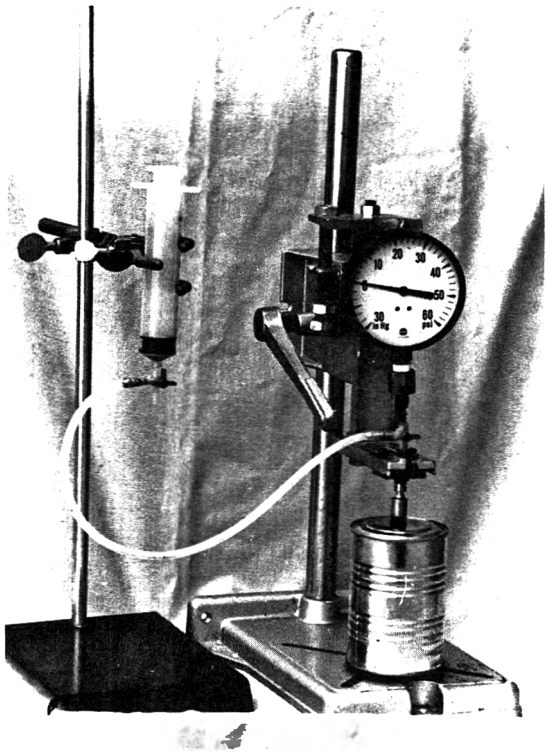

FIG. 46:02—Puncturing press

× ½ in. silicone rubber gasket around beveled ⅛ in. piercer to maintain seal.

(c) *Helium exposure tank.*—ASME paint tank, 10 gal., tested to 100 psi, equipped with inlet and outlet microcontrol valves (Harrison Rubber and Supply Co., Court and Race Sts, Cincinnati, OH 45202).

(d) *Pressurized helium tank.*—With 2 stage regulator.

(e) *Timer and solenoid.*—To automate release of He from exposure tank.

(f) *Helium gas standards.*—Scientific Gas Products, Inc., 2330 Hamilton Blvd, South Plainfield, NJ 07090.

(g) *Cyanoacrylate glue.*—Superglue (3M, Minneapolis, MN, or equiv.).

(h) *Can opener.*—Bacteriological, **46.065**(a).

(i) *Rubber discs.*—2⅜ in. × ⅛ in. and 70 durometer (Netherland Rubber Co., 629 Burbank, Cincinnati, OH 45206).

Calibration Test Procedure

For gas chromatographs equipped with side port loop (0.5 mL), inject 5.0 mL calibrated He stds (suggested range of 5, 15, 25, 50, and 75% He). For instruments not equipped with side port loop, inject appropriate vol. of stds. Use same vol. for analysis of headspace gas samples. Plot percent He vs He peak ht at attenuation used. Depending on qual. of instrument, plot should approx. a straight line.

Check gage on can piercer against known pressure and vac.

Test resealing procedure, **46.075,** on control cans.

Helium Exposure Tanl

Control introduction rate of He into exposure tank, and time cans are exposed to He pressure at 45 ± 2 psi. Timer, solenoid, and microvalves with vernier scales can facilitate procedure. Connect He source to exposure tank. Turn timer on to close outlet solenoid valve. Approx. 15–20 min are needed to reach 45 psi in tank. Make minor adjustments if necessary. Adjust timer to expose cans to He pressure at 45 psi for 30 min (30 min exposure period is in addition to time necessary to reach 45 psi). Tank pressure should be reduced to 0 psi within 5–10 min.

Preparation of Can for Helium Test

If sample is to be taken for microbiological testing, proceed as in **46.066.**

For nonsterile opening of can, use opener, **46.065**(a) to cut 1½ in. hole in can lid. Remove and discard portion of contents.

Push down any sharp metal projections around 1.5 in. hole. Wipe lid dry and lightly sand area where rubber disc will be inserted. Pool cyanoacrylate glue around surface covered by edge of rubber disc. Place disc over hole and smooth edges with fingers to remove air bubbles. Place wt (>500 g) on disc ≥ 1 h.

Collection and Headspace Gas Analysis

Can piercing assembly is shown in Fig. **46:02.** Before piercing can, close gage valve and pull plunger on syringe to remove air from silicone tubing. Close syringe valve and expel air from syringe. Puncture can and open gage valve to read vac. or pressure. Turn gage valve and syringe valve to release gas into syringe. If gas sample is >5.0 mL, withdraw this amt (as shown in Fig. **46:02**) and inject into port of gas chromatograph. If gas sample is <5.0 mL, force collected gas back into can. Close syringe valve to retain gas in tubing and can. Use syringe to add 40 mL room air to can, and pump syringe twice to mix gas. Let syringe equilibrate to atm. pressure and record syringe vol. From this dil. gas, sample may be obtained for gas chromatograph. Percent He measured should be divided by diln factor to obtain correct percent He in headspace gas. Use following formula to det. diln factor:

$$\text{Diln factor} = (\text{equilibrated syringe vol.} - 40\text{ mL air} + \text{headspace vol.})/(\text{equilibrated syringe vol.} + \text{headspace vol.})$$

For example: (43 − 40 + 9)/(43 + 9) = 12/52 = 0.23 diln factor

$$\%\text{ He in can} = \%\text{ He measured/diln factor}$$

For example: 5% He/0.23 = 22% He in can

Headspace vol. may be measured by piercing control can that still has vac. Assume sample and control can same volume. Measure amt of vac. (in. Hg) and vol. air pulled in from syringe.

$$\text{Headspace vol.} = \text{measured vol. from syringe} \times 30\text{ in. Hg/measured vac. in can (in. Hg)}$$

For example, if 6 mL air is pulled into can and vac. is 20 in. Hg, then:

$$\text{Headspace vol} = 6\text{ mL} \times 30\text{ in. Hg}/20\text{ in. Hg} = 9\text{ mL}$$

To perform addnl work on can, collected gas may be stored in capped syringe 2–3 h without appreciable change in its composition.

Interpretation of Results

Report can as leaker if, after exposure to pressurized He, can internal pressure is ≥8 psi or percentage He is ≥1%. Report can as nonleaker if, after exposure to pressurized He, can internal vac. is ≥5 in., or percentage He is <1%.

Table 1. Number of can leaks observed by the vacuum and helium tests

Can type	Coll.	Vacuum test				Helium test				Total number of cans tested
		I[a]	II	II	Total	I	II	II	Total	
Neg. control	1	0	0	0	0	0	1	0	1	15
Nondented	2	0	0	0	0	0	0	0	0	15
Flat cans	3	0	0	0	0	0	0	0	0	15
Flat cans	4	0	0	0	0	0	0	0	0	15
Flat cans	5	0	0	0	0	0	0	0	0	13
Flat cans	6	1	0	0	1	0	0	0	0	15
Flat cans	7	0	0	0	0	0	0	0	0	14
Total		1	0	0	1	0	1	0	1	102
		I	III	III	Total	I	III	III	Total	
Neg. control	1	0	0	0	0	0	0	1	1	13
Nondented	2	0	0	0	0	1	1	0	2	15
Swollen cans	3	0	0	0	0	0	0	0	0	15
Swollen cans	4	0	0	0	0	0	0	1	1	15
Swollen cans	5	0	0	0	0	0	5	2	7	15
Swollen cans	6	0	0	0	0	0	0	0	0	15
Swollen cans	7	0	—[b]	—[b]	0	1	—[b]	—[b]	1	4
Total		0	0	0	0	2	6	4	12	92
		I	II	II	Total	I	II	II	Total	
Neg. control	1	5	9	5	19	7	8	5	20	26
Dented cans	2	5	8	4	17	6	10	8	24	30
Flat cans	3	7	5	8	20	10	6	7	23	29
Flat cans	4	4	0	0	4	7	10	9	26	29
Flat cans	5	3	5	6	14	6	6	9	21	29
Flat cans	6	8	8	9	25	7	8	9	24	30
Flat cans	7	2	7	4	13	9	8	8	25	27
Total		34	42	36	112	52	56	55	163	200
		I	III	III	Total	I	III	III	Total	
Neg. control	1	2	3	3	8	2	9	8	19	25
Dented cans	2	0	7	2	9	1	10	8	19	23
Swollen cans	3	2	3	4	9	6	4	5	15	26
Swollen cans	4	6	5	6	17	8	8	6	22	30
Swollen cans	5	0	10	2	12	3	10	4	17	27
Swollen cans	6	6	3	2	11	1	2	1	4	29
Swollen cans	7	2	—[b]	—[b]	2	8	—[b]	—[b]	8	9
Total		18	31	19	68	29	43	32	104	169

[a]Sequence in which the samples were analyzed.
[b]Samples not analyzec.

Table 2. Summary of can leaks detected by food product and data set

Can type	Data set	Food type	No. of leaks: Vacuum test	No. of leaks: Helium test
Nondented	I[a]	Creamed-style corn	1	0
Flat cans	II	Carrots	0	1
Flat cans	II	Green beans	0	0
Nondented	I	Creamed-style corn	0	2
Swollen cans	III	Fruit cocktail	0	6
Swollen cans	III	Whole-kernel corn	0	4
Dented cans	I	Creamed-style corn	34	52
Flat cans	II	Carrots	42	56
Flat cans	II	Green beans	36	55
Dented cans	I	Creamed-style corn	18	29
Swollen cans	III	Fruit cocktail	31	43
Swollen cans	III	Whole-kernel corr	19	32

[a]Sequence in which the samples were analyzed.

Results and Discussion

More leaks were detected with the helium leak test than with the vacuum test for flat and swollen cans and for all food products. Only 1 collaborator observed more leaks with the vacuum leak test than with the helium test.

Three sets of cans were sent to the 7 collaborators. The first set consisted of flat and swollen dented cans plus nondented controls. A similar set of samples was sent in the second set. Only the results from flat cans were returned, because it was difficult to prepare swollen cans. The swollen cans were discarded, and a third group of cans consisting of swollen dented cans and controls was shipped. Table 1 shows results from the study, and Table 2 summarizes these results by food product. I, II, and III denote the sequence of analysis. The number of positive results (leakers) is tabulated in these tables.

Table 1 shows the number of cans that could be used to compare vacuum and helium tests. The 15 controls and 30 dented cans sent to replace the flat and swollen cans represent the maximum number of comparative results. However, experimental problems prevented the completion of one or both of the tests. Collaborator 7 did not report 36 tests.

Three collaborators experienced particular problems with resealing the cans during the analysis of the first set. The technique involving swollen cans was sensitive to surface

Table 3. Summary of results from each collaborator

Coll.	% positive leakers[a] Vacuum test	% positive leakers[a] Helium test	Chi-square X_1^2	Total No. of cans tested
3	31.1	44.7	1.58	85
6	41.6	31.5	1.55	89
1	34.2	51.9	4.36[b]	79
2	31.3	54.2	7.97[b]	83
5	31.0	53.6	7.90[b]	84
4	23.6	55.1	17.16[b]	89
7	27.8	63.0	12.10[b]	54

[a]Cans with reported values of ≥ 1% were noted as leakers.
[b]Significant at the $\alpha = 0.05$ level.

Table 4. Comparison of test procedures for can type

Can type	% positive leakers[a] Vacuum test	% positive leakers[a] Helium test	Chi-square X_1^2	No. of cans examined
Nondented flat cans	1.0	1.0	0.00	102
Dented flat cans	56.0	81.5	29.09[a]	200
Nondented swollen cans	0.0	13.0	10.79[a]	92
Dented swollen cans	40.2	61.5	14.50[a]	169

[a]Significant at the $\alpha = 0.05$ level.

preparation and sealant application during insertion of the rubber disc. The results from the third set of cans indicated that the sealing was adequate. Of 630 cans, 563 were available for analysis. Of the 67 cans for which results could not be obtained, 30 were from Collaborator 7, who could not analyze the sample because of scheduling problems. The 37 (6.2%) other missing results were due to resealing problems, valve leaks, excess end distortion, and lack of reporting (3 tests).

Table 3 presents a statistical summary of results by collaborators. Each collaborator received 90 cans. The percent of positive leakers was computed for each test by collaborator. Collaborator 6 found more positive results with the vacuum test. Five of the 7 collaborators had significantly ($\alpha = 0.05$) higher results with the helium test. The chi-square test (5) was performed on a 2 × 2 contingency table (2 tests, 2 results). Table 4 lists percent positive leakers by type of can (flat and swollen). Only 1% of the flat controls were positive, but 13% of the swollen controls were positive. Collaborator 5 had 7 of the 12 positive results.

The helium test yielded significantly ($\alpha = 0.05$) more positive results on dented cans than did the vacuum test. Of the 400 dented cans sent as possible leakers, the helium test found 267 positives, and the vacuum test found 181. With the dented flat cans, positive results were 81.5% for the helium versus 56% for the vacuum test. For dented, swollen cans, positive results were 61.5% for the helium and 40.2% for the vacuum test.

All of the dented cans were expected to have leaks, but preliminary experiments showed that some of these leaks resealed or that food particles clogged the holes. Thus, it was impossible to send homogeneous samples to the collaborators. The number of cans that still leak when they reach the collaborators is not known, but 20% fewer positives were detected in swollen cans than in flat dented cans. The internal pressure may produce resealing in more of the swollen cans. Results from our laboratory indicate that holes as small as 1 μm in diameter can be detected by the helium test. Leaks in dented cans are irregular in shape, and, thus, a minimum limit of detectability would be difficult to specify.

The collaborators reported the pressure on the gauge when the cans were pierced. When the gauge pressure was ≥ 8 psi, the helium test yielded a positive result. Fifty-eight percent of the flat leakers and 71% of the swollen leakers had ≥ 8 psi reading. The time required for the helium test can be decreased by reporting a can with gauge pressure ≥ 8 psi as positive and omitting the gas chromatographic analysis.

Resealing problems were evident in the first set of samples, but the third group seemed to confirm the utility of the test. Though the vacuum test can be performed in less time, the helium test can detect more leaks and is less subjective in interpretation. We recommend that the helium leak test be adopted official first action.

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Detection of *Escherichia coli* Enterotoxins by Using Mouse Adrenal Cell and Suckling Mouse Assays: Collaborative Study

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The ability of 10 *Escherichia coli* strains to produce heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) was determined by 8 analysts in a collaborative study. The suckling mouse model and the mouse adrenal cell line (Y-1) tests were used to detect ST and LT, respectively. Cultures for assay were grown 24 h in casamino acid–yeast extract–trace salts broth at 37°C in a shaker incubator at 250 rpm. Cell-free culture broth prepared by centrifugation and filtration was divided into 2 portions: One was heated for 30 min and used both for ST assay and as a heated control for LT assay; the other was used unheated for LT assay. Results were expressed as positive for ST, positive for LT, positive for ST and LT, or negative for both ST and LT; percent of correct estimates was calculated for each culture for each analyst. At the 95% confidence interval, the overall correct results were 96.3 ± 2.9 and 95.0 ± 3.4% for ST and LT, respectively. The test performances thus were satisfactory for detecting ST and LT produced in vitro by *E. coli*. The method has been adopted official first action.

When exposed to cholera toxin or the heat-labile enterotoxin (LT) of *Escherichia coli*, the Y-1 mouse adrenal cell line responds by a change in morphology from flat to round. This response is mediated by adenyl cyclase and is irreversible (1, 2). The intragastric administration of heat-stable enterotoxin (ST) of *E. coli* to the suckling mouse causes fluid accumulation in the intestinal lumen. This measurable response is mediated by guanyl cyclase (3, 4). Although both of these assays have been in common use for a decade, neither has been studied collaboratively. The purpose of this study was to determine the suitability of using these 2 assays to detect *E. coli* enterotoxins.

Interlaboratory Study Protocol

Cultures

Each of 8 analysts was supplied with 10 *E. coli* strains in duplicate, numbered 1–20. The strains and the enterotoxins produced by them are shown in Table 1. Cultures were maintained and shipped to collaborators on trypticase soy broth. Known enterotoxin-positive and -negative cultures, previously supplied as a practice set, were recommended as positive and negative culture controls. In addition, each analyst was supplied with the tissue culture for the LT assay, a copy of the protocol, analyst's worksheets, and a report form. Copies of the worksheets and the original report form were returned when the assays were completed. The Y-1 mouse adrenal cell line was obtained from American Type Culture and was maintained and shipped to the collaborators in a growth medium described later.

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The recommendation of the Associate Referee was approved by the General Referee and Committee F and adopted by the Association. See *J. Assoc. Off. Anal. Chem.* (1984) **67**, March issue.

Escherichia coli Enterotoxins Mouse Adrenal Cell and Suckling Mouse Assays First Action

Principle

When exposed to cholera toxin or heat-labile enterotoxin of *Escherichia coli*, mouse adrenal cell line, designated Y1, responds by change in morphology from flat to round. Response is mediated by adenyl cyclase and is irreversible. Intragastric administration of heat-stable enterotoxin of *E. coli* to suckling mouse causes fluid accumulation in intestinal lumen. This measurable response is mediated by guanyl cyclase.

Media and Reagents

(a) *Casamino acids-yeast extract (CAYE) broth.—Soln a:* Casamino acids, 20 g; yeast ext, 6 g; NaCl, 2.5 g; K_2HPO_4, 8.71 g; adjust to pH 8.5 with 0.1N NaOH, and to final vol. of 1 L. *Soln b:* $MgSO_4$, 50 g; $MnCl_2$, 5 g; $FeCl_2$, 5 g; dissolve in min. amt of 0.01N H_2SO_4, and adjust to final vol. of 1 L with H_2O.

Add 1 mL soln b to soln a before sterilizing; autoclave 15 min at 121°C after dispensing.

(b) *Trypticase soy-yeast extract (TSYE) broth.*—Com. trypticase soy broth rehydrated as directed with 0.6% yeast ext added.

(c) *Tissue culture media.—(1) Growth medium:* Ham's F-10 with glutamine and $NaHCO_3$ (Flow Labs), 100 mL; newborn calf serum, 10 mL; antibiotic conc. (5000 IU penicillin G, and 5000 μg streptomycin/mL), 1 mL. *(2) Maintenance medium:* Same as *(1)* except serum level is 1%.

(d) *Dulbecco's PBS, pH 7.5.*—NaCl, 8 g; KCl, 200 mg; $Na_2HPO_4.7H_2O$, 2.16 g; KH_2PO_4, 200 mg; make up to 1 L with H_2O and autoclave 15 min at 121°.

(e) *Trypsin.*—0.25% in Dulbecco's PBS.

(f) *Cholera enterotoxin.*—1 mg/mL when reconstituted as directed (Schwartz/Mann).

(g) *Mice.*—Outbred white Swiss mice, 3–5 days old.

(h) *Evans blue.*—2% soln.

Equipment and Materials

(a) *Serological pipets.*—1 and 5 mL, small tip.

(b) *Pipets.*—25 μL.

(c) *Swinnex filters.*—25 mm 0.45 μm membrane.

(d) *Disposable syringes.*—5 mL, accommodating Swinnex filters.

(e) *Tissue culture flasks.*—Plastic, 75 sq. cm.

(f) *Vertical laminar flow hood.*—Biological containment, equipped with HEPA filters.

(g) *Incubator.*—CO_2, set at 35° and 5% CO_2.

(h) *Microtiter tissue culture plates.*—96 wells, with lids, sterile.

(i) *Syringe.*—1 mL, disposable.

(j) *Animal feeding needle.*—24 gage, 1 in., straight.

(k) *Needle.*—27 gage. Not needed if *per os* procedure is followed.

Table 1. Cultures used in *E. coli* enterotoxins bioassay collaborative study

Culture			
Nc.	Identity	Source	Enterotoxins produced
1 and 19	1362	H. W. Moon[a]	LT and ST
2 and 14	B7A	I. Mehlman[b]	LT and ST
3 and 17	263	H. W. Moon	LT only
4 and 15	1408	H. W. Moon	LT only
5 and 20	B44	I. Mehlman	ST only
6 and 13	E38	FDA Collection	none
7 and 18	987	H. W. Moon	ST only
8 and 12	H10407	I. Mehlman	LT and ST
9 and 16	E19	FDA Collection	none
10 and 11	339	I. Mehlman	LT and ST

[a]USDA, National Animal Disease Laboratory, Ames, IA 50010.
[b]Division of Microbiology, FDA, Washington, DC 20204.

Labile Toxin (LT)

Day 1

(a) Inoculate control cultures and cultures to be assayed into TSYE broth in 16 × 125 mm screw-cap tubes. Incubate in shaker incubator overnight at 37°. Both known enterotoxin-pos. and enterotoxin-neg. *E. coli* cultures should be used as controls, in addition to cholera toxin-pos. control.

(b) Remove growth medium from confluent layer of Y1 cells in 75 sq. cm flask. (One flask will provide enough cells for 2 microtiter assay plates.) Wash cell layer with PBS. Remove PBS wash and add 5 mL trypsin. After 1 min exposure, remove 4.5 mL trypsin and place flask in 35° incubator. Observe at 5 min intervals for cell detachment. When cell sheet has detached, add 5 mL growth medium and pipet repeatedly to break up cell clumps.

(c) Add cells from Day 1 (b) to 35 mL growth medium (total vol. is 40 mL) in small beaker. Stir this suspension while pipeting 0.2 mL into each well of two 96-well microtiter plates, using macroliter pipet. Cover finished plates and incubate ca 48 h at 35° in CO_2 incubator.

Day 2

(d) Add 2 drops of previously prepd starter culture, Day 1 (a), to 10 mL CAYE broth in 50 mL Erlenmeyer and incubate 24 h at 37° in shaker incubator at 250 rpm.

Day 3

(e) Centrf. 24 h culture from Day 2 (d). (Twenty min at 2500 rpm will clarify most cultures of *E. coli*.). Filter supernate thru 0.45 μm membrane in Swinnex syringe-end filter holder.

(f) Divide filtrate into 2 portions. Heat one portion 30 min at 80°; leave other portion unheated. Both heated and unheated portions are assayed. Heated portion serves as neg. control. Store both at 4°.

(g) Prep. cholera toxin soln of 1 ng CT/mL in PBS. Soln is used as pos. control for cell reactivity. Note: CT is unstable at this concn, even at 4°; prep. daily from stock soln.

(h) Remove microtiter plates prepd in Day 1 (c) and replace growth medium with maintenance medium, 0.2 mL/well.

(i) Add 0.025 mL assay and control solns to one or more wells (4/test substance recommended) of microtiter plate, using microtiter syringe. Incubate microtiter plates 30 min at 35° in CO_2 incubator.

Replace maintenance medium, 0.2 mL/well. Incubate microtiter plates overnight at 35° in CO_2 incubator.

Day 4

(j) Examine microtiter plates for degree of rounding, starting with controls. Score rounding as follows:

0 = no rounding
1 = ca 25% rounding
2 = ca 50% rounding
3 = ca 75% rounding
4 = 100% rounding

Score ≥2 is reported as pos. for LT. Score <2 is recorded as neg. Neg. controls should show <10% rounding.

Stable Toxin (ST)

Day 1

(a) Inoculate starter culture. Procedure is identical to Day 1 (a), and need not be repeated when both assays (LT and ST) are done concurrently.

Day 2

(b) Inoculate CAYE assay culture. Procedure is identical to Day 2 (d), and need not be repeated when both assays (LT and ST) are done concurrently.

Day 3

(c) Prep. cultures for assay. Procedure is same as Day 3 (e) and (f), and need not be repeated if both assays (LT and ST) are done concurrently. Heated portion only is used in ST assay (suckling mouse). Material for ST assay may be stored at 4° for several days without noticeable loss of activity.

Day 4

(d) Add 2 drops of sterile Evans blue to 1 mL filtrate to be assayed.

(e) Inject suckling mice percutaneously with 0.1 mL filtrate into milk-filled stomach. Use tuberculin syringe and 27 gage needle. Inject min. of 4 mice for each filtrate. Discard all injections in which blue filtrate is not confined to stomach (immediate visual inspection) *or*

(f) Inject *per os* 0.1 mL filtrate into stomach of each mouse, using tuberculin syringe equipped with 24 gage feeding needle. This procedure may be used instead of percutaneous injection described in Day 4 (e). Either method works and preference is based on analyst's familiarity. Both methods yield equiv. results.

(g) Hold mice 3 h at *room temp*. Sacrifice mice by CO_2 inhalation. Open each abdomen and remove intestinal tract with exception of stomach and liver. Pool intestines treated with same filtrate in tared weighing vessel. Pool remainder of carcasses in another tared weighing vessel. Weigh both vessels on balance accurate to 0.01 g. Compute ratio of intestine wt/carcass wt.

Interpretation

Report ratio ≥0.083 as pos. for ST. Report ratio ≤0.074 as neg. for ST. Ratio 0.075–0.082 calls for re-examination of filtrate involved.

Results and Discussion

At 2 steps in the assay protocol, the analyst was given options. In culturing bacteria for assay, either a shaker incubator or roller tube device was permitted for culture aeration. Although the roller tube device was not preferred, it was allowed in deference to those laboratories without shaker incubators. Only one analyst, No. 7, used the roller tube device. The second option, either percutaneous or per os inoculation of infant mice, was permitted because some analysts had learned and preferred one or the other procedure. Our experience indicated that both procedures gave equiva-

Table 2. Results of *E. coli* enterotoxins bioassay collaborative study

Sample	*E. coli* strain	Expected result LT	Expected result ST	Coll. 1 LT	Coll. 1 ST	Coll. 2 LT	Coll. 2 ST	Coll. 3 LT	Coll. 3 ST	Coll. 4 LT	Coll. 4 ST	Coll. 5 LT	Coll. 5 ST	Coll. 6 LT	Coll. 6 ST	Coll. 7 LT	Coll. 7 ST	Coll. 8 LT	Coll. 8 ST
1	1362	≥2.0	≥0.083	4.00	0.126	2.75	0.088	4.00	0.103	0.00	0.052	3.00	0.086	4.00	0.117	4.00	0.089	4.00	0.111
2	B7A	≥2.0	≥0.083	4.00	0.120	3.50	0.091	4.00	0.196	3.00	0.116	3.00	0.093	4.00	0.124	1.00	0.060	4.00	0.145
3	263	≥2.0	<0.075	4.00	0.068	1.75	0.057	3.00	0.064	3.00	0.068	3.50	0.052	4.00	0.068	4.00	0.059	4.00	0.074
4	1408	≥2.0	<0.075	4.00	0.072	3.00	0.063	4.00	0.057	3.00	0.055	3.50	0.057	3.50	0.064	4.00	0.064	4.00	0.056
5	B44	<2.0	≥0.083	0.00	0.119	0.00	0.095	0.00	0.129	0.00	0.117	0.25	0.104	1.00	0.139	0.00	0.101	0.75	0.127
6	E38	<2.0	<0.075	0.00	0.072	0.00	0.065	0.00	0.052	0.00	0.064	0.00	0.054	1.00	0.062	0.00	0.061	0.50	0.062
7	987	<2.0	≥0.083	0.00	0.119	0.00	0.087	0.00	0.110	0.00	0.104	0.50	0.108	1.50	0.106	0.00	0.085	0.00	0.134
8	H10407	≥2.0	≥0.083	4.00	0.125	3.75	0.095	3.00	0.097	4.00	0.096	2.75	0.096	4.00	0.085	4.00	0.062	4.00	0.063
9	E19	<2.0	<0.075	0.00	0.064	0.00	0.060	0.00	0.057	0.00	0.043	0.00	0.054	1.00	0.064	0.00	0.056	0.00	0.065
10	339	≥2.0	≥0.083	3.00	0.112	3.25	0.102	3.00	0.124	4.00	0.107	0.00	0.140	4.00	0.105	3.00	0.092	4.00	0.121
11	339	≥2.0	≥0.083	2.00	0.126	1.75	0.106	3.00	0.073	2.00	0.124	0.00	0.121	4.00	0.102	4.00	0.098	4.00	0.120
12	H10407	≥2.0	≥0.083	3.00	0.092	2.75	0.126	3.00	0.119	3.00	0.102	1.00	0.105	4.00	0.071	1.00	0.054	3.00	0.070
13	E38	<2.0	<0.075	0.00	0.060	0.00	0.053	0.00	0.062	0.00	0.042	1.50	0.062	0.00	0.060	0.00	0.084	0.00	0.053
14	B7A	≥2.0	≥0.083	4.00	0.137	4.00	0.105	3.00	0.119	2.50	0.100	2.50	0.113	4.00	0.117	0.00	0.089	3.00	0.155
15	1408	≥2.0	<0.075	4.00	0.072	2.75	0.057	3.00	0.065	3.00	0.039	3.00	0.058	4.00	0.058	0.00	0.047	3.00	0.065
16	E19	<2.0	<0.075	1.00	0.069	0.00	0.056	0.00	0.052	0.00	0.036	0.00	0.058	0.00	0.054	0.50	0.091	0.00	0.053
17	263	≥2.0	<0.075	4.00	0.070	0.25	0.056	2.00	0.057	3.00	0.048	2.75	0.052	4.00	0.054	3.75	0.054	3.00	0.083
18	987	<2.0	≥0.083	0.00	0.096	0.00	0.103	0.00	0.102	0.00	0.111	0.25	0.111	1.00	0.090	0.00	0.091	0.00	0.143
19	1362	≥2.0	≥0.083	4.00	0.089	1.50	0.102	2.00	0.098	2.00	0.114	2.50	0.092	4.00	0.102	4.00	0.110	3.00	0.136
20	B44	<2.0	≥0.083	0.00	0.117	0.00	0.113	0.00	0.102	0.00	0.108	1.50	0.101	2.00	0.102	0.00	0.063	0.00	0.111
Culture method				shaker		shaker		shaker		shaker		shaker		shaker		roller tube		shaker	
Mouse inoculum route[a]				pc		po		pc		po		po		po		pc		pc	

[a]pc = percutaneous; po = per os.

Table 3. Summary of *E. coli* ST bioassay results

Strain	Mean of result	CV, %[a]	Correct result, %[b]
1362	0.101[c]	20.3	94
B7A	0.118	26.9	94
263	0.062	14.1	94
1408	0.060	14.0	100
B44	0.109	14.8	94
E38	0.060	16.5	94
987	0.106	16.0	100
H10407	0.091	25.5	69
E19	0.058	21.0	94
339	0.111	14.8	94

[a]Percent coefficient of variation of error plus among-analysts components (5).
[b]Mean percent correct result with 95% confidence interval = 92.5 ± 4.1.
[c]Computed gut weight/body weight ratios, suckling mouse assay.

Table 4. Summary of *E. coli* LT bioassay results

Strain	Mean of result	Correct result, %[a]
1362	3.05[b]	94
B7A	3.09	88
263	3.13	88
1408	3.23	94
B44	0.34	94
E38	0.19	100
987	0.20	100
H10407	3.14	88
E19	0.16	100
339	2.81	81

[a]Mean percent correct result with 95% confidence interval = 92.5 ± 4.1.
[b]Based on visual estimate of Y-1 rounding, scored on a scale of 0–4.

lent results. Four analysts chose percutaneous and 4 chose per os routes for the inoculum.

Data resulting from both the LT and ST assays are given in Table 2. ST values are means of calculated ratios of gut weight over body weight of all mice given a particular culture inoculum. These measurements were subjected to analysis of variance procedures. LT values are the means of 4 visual estimates of percent of Y-1 cell rounding for each culture fluid, converted to a score varying from 0 to 4. LT bioassay scores are more subjective and qualitative, and therefore were not subjected to analysis of variance procedures. Components of variance of ST measurements were estimated by analysis of variance (5) for each culture in the suckling mouse assay procedures. However, both ST and LT assays, collaboratively studied as screening tools, are qualitative tests designed to say yes or no to the question: Does this culture produce ST and/or LT? Since each culture had a history of consistent enterotoxin production or lack of it, cultures were expected to yield either positive or negative results. The percentage of correct results (CR) was calculated for each culture and each analyst.

Table 3 presents mean results of the ST bioassay with percent coefficient of variation (CV), and percent CR by culture. Estimates of percent CV for components for ST ranged from 14.0 to 26.9%. The components on which these variance estimates are based consist of experimental error and among-analyst error (5). Values of percent CR for ST ranged from 94 to 100 for 9 of 10 cultures. A value of 69%, observed for culture H10407, resulted from the reports of 3 analysts. The overall percent CR with 95% confidence interval for ST bioassay was 92.5 ± 4.1.

Table 4 presents the mean results of LT bioassay with percent CR. The overall percent CR with 95% confidence interval for LT bioassay was also 92.5 ± 4.1.

The percent CR (by analyst) for both bioassays (ST and LT) is given in Table 5. Analyst 7 had significantly ($\alpha = 0.05$) lower gut weight/body weight values and thus had only 70% CR for the ST bioassay. Of 12 false scores for ST, 6 came from analyst 7's report. Of 12 false scores for LT, 4 were contributed by analyst 7. If percent CR were recalculated for both bioassay procedures, eliminating the data of analyst 7, the values for ST and LT with 95% confidence intervals would be 96.3 ± 2.9 and 95.0 ± 3.4%, respectively.

We believe the poor performance of analyst 7 was due to the choice of a roller tube device to provide culture aeration. This analyst was the only one to make that choice, and the dissimilarity of those results is striking. We have concluded that roller tube aeration is not adequate and recommend the use of a shaker incubator at 250 rpm for culture production as the only acceptable procedure.

Two hypotheses remain to be tested: (1) the 2 tests being evaluated identified known enterotoxin negative and entero-

Table 5. Summary of *E. coli* enterotoxins bioassay results: analyst performance

Analyst	ST bioassay Correct result, %	LT bioassay Correct result, %
1	100	100
2	100	85
3	95	100
4	95	95
5	95	85
6	100	95
7	70	80
8	85	100
Mean	92.5	92.5
Mean without analyst 7	96.3	95.0

toxin positive cultures at the same frequency, and (2) per os and percutaneous infant mouse inoculation gave equivalent results. To test these hypotheses, estimates of overall proportion of correct identification were computed. The proportions (p) were 1.00 and 0.92 for known negative and positive LT cultures, and 0.94 and 0.92 for known negative and positive ST cultures, respectively. These proportions do not differ significantly at the $\alpha = 0.05$ level (6). When ST results were examined for differences in data generated by the 2 mouse inoculation methods, only results from the 7 collaborators who used a shaker incubator for culture preparation were used. The proportion of correct results was 0.93 for per os and 0.98 for percutaneous inoculation methods. These proportions do not differ significantly ($\alpha = 0.05$) (6). Therefore, evidence to reject the 2 hypotheses previously stated is insufficient.

Performance of the bioassay procedure, analyzed qualitatively and with or without the data of analyst 7, was satisfactory. We recommend, therefore, that the suckling mouse assay for *E. coli* ST and the Y-1 tissue culture assay for *E. coli* LT be adopted official first action for the screening of *E. coli* isolates for enterotoxigenicity. We also recommend that the LT and ST bioassay protocols be modified to remove all reference to the roller tube device in culture preparation and that the methods, as modified above, be adopted official first action for the presumptive recognition of *E. coli* enterotoxigenicity.

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Development of an Overnight Rapid Bovine Identification Test (ORBIT) for Field Use

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An Overnight Rapid Bovine Identification Test (ORBIT) has been developed as a serological screen test for species verification of raw, whole tissue, bovine meat products. The test, an agar-gel immunodiffusion technique, uses stabilized reagent paper discs and prepared agar plates that have a printed template for correct placement of test components. This test is reliable, practical, economical, and easily performed in the field, such as at a meat import inspection station. The only nonbovine species found to react in the test are the bovine-related species of American bison (buffalo) and water buffalo (from Australia); however, these rare-occurring species do not present a problem for the intended application of the test. Stability of all test components, when stored in a refrigerator, is excellent for at least 1 year. The nature and stability of the test make it suitable for commercial development into test kits which should be highly practical and economical for wide availability and application of this procedure to meat inspection programs concerned with species verification.

The Food Safety and Inspection Service of the U.S. Department of Agriculture is responsible for the assurance of a safe, wholesome, unadulterated, and accurately labeled meat and poultry supply to consumers. Legislative authority for this mandate originates from the Meat Inspection Act of 1906 and the Federal Poultry Products Inspection Act of 1957, with specific regulations for implementation cited in Title 9 of the *Code of Federal Regulations* (1). The agency carries out this mission through inspection, conducting laboratory analytical services, industrial monitoring programs, and compliance (enforcement) programs on domestic and imported meat, poultry, and meat and poultry food products. One important aspect of laboratory analysis concerns the accurate identification of the species of animal tissue used in consumable meat products to assure that there is no adulteration or fraudulent substitution.

Species identification of animal tissue can generally be performed by traditional immunological methods such as the interfacial ring precipitin test (2, 3) or agar-gel immunodiffusion (4, 5), both of which use specific antispecies sera and saline extracts of tissue. Other current approaches to species determination of meat, as well as fish tissue, include techniques such as enzyme-linked immunosorbent assay (6, 7), radioimmunoassay (8), polyacrylamide gel electrophoresis (9), and thin layer isoelectric focusing in agarose (10) or

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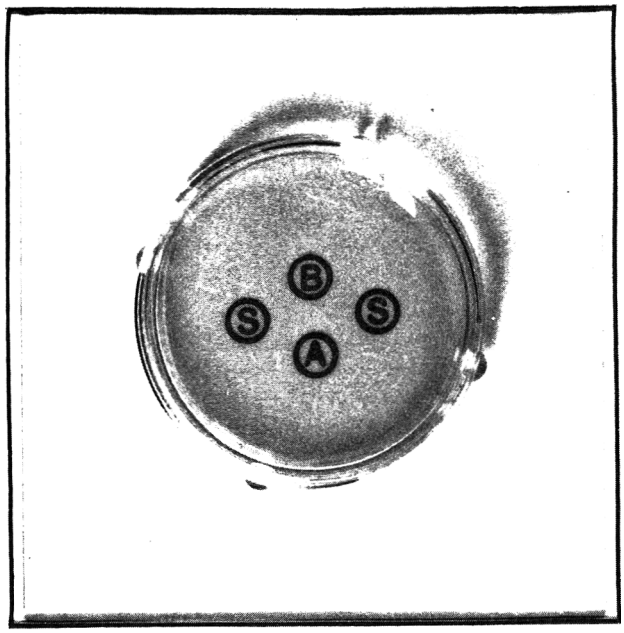

Figure 1. ORBIT plate with printed pattern for standardized disc placement on agar surface. Lettered circles are 6.5 mm in diameter and 5 mm equidistant with respect to their adjacent inner edges. The 2 sample circles (S) are 13.5 mm directly opposite each other.

polyacrylamide gel (11). These procedures, although accurate in their ability to determine species, have disadvantages, such as the need for performance in a formal laboratory, use of expendable, labile biochemical reagents that require special care for maintenance, expensive equipment, and a requirement for technical expertise on the part of the individual who performs the test and interprets the results. Use of such methods also incurs a relatively high cost factor per sample analyzed due to the additional expense and time delay associated with sample shipment to the laboratory and reporting final results. When large numbers of samples must be analyzed for regulatory purposes, a high cost factor is undesirable and becomes a limiting factor in surveying for potential adulteration or substitution problems.

Because of these problems and limitations, an accurate species identification system is needed, which is rapid, easy to perform and interpret, cost-effective per sample analysis, suitable for use in a field setting, such as an abattoir, meat establishment, or import inspection station, and which can be provided in a kit that contains the necessary materials as stabilized components. Beef is one of the leading meats consumed and imported throughout the world, so development of a reliable screen test for bovine species, which meets the above requirements, is highly desirable. We have studied the application of the agar-gel immunodiffusion technique, using reagent-impregnated filter paper, previously reported (12) for in vitro determination of bacterial-isolate toxigenicity, to the solution of this problem. In this paper, we report a test system, referred to as the Overnight Rapid Bovine Identification Test (ORBIT), which meets all of the objectives mentioned and which is intended for use on raw, single, whole-muscle tissue samples.

METHOD

Apparatus

(a) *Petri dish.*—Falcon No. 1006 tight lid, 50 × 9 mm (Falcon, Div. of Becton, Dickinson & Co., Oxnard, CA), custom silk-screen printed (BMD, Inc., Savage, MD) with 4 lettered circles in pattern (Figure 1) on outside of bottom plate.

(b) *Filtering cloth.*—Miracloth (Calbiochem-Behring, San Diego, CA).

(c) *Paper discs.*—BBL No. 31039 blank filter paper sensitivity test discs (Becton Dickinson and Co., Cockeysville, MD).

(d) *Lyophilizer.*—Freezemobile II lyophilizer with Model 10-MR-SA single shelf, vacuum drying chamber (Virtis, Gardiner, NY).

(e) *Stomacher.*—Colworth Model 80 stomacher (Tekmar Co., Cincinnati, OH).

(f) *Bacteriological filter.*—Millipore Millex-GS, 0.22 μm filter unit (Millipore Corp., Bedford, MA).

(g) *Plate reader.*—Hyperion viewer with magnifier, Model No. 4040-100A (Hyperion, Inc., Miami, FL).

(h) *Extraction bag.*—Whirl-pak polyethylene bag, 22.8 × 11.4 cm (Nasco, Fort Atkinson, WI).

Reagents

(a) *Agar.*—Difco agar, purified, No. 0560-01 (Difco Laboratories, Detroit, MI).

(b) *Phosphate-buffered saline.*—0.85% NaCl solution containing 1.25 mL/L of stock 0.25M KH_2PO_4 solution previously adjusted to pH 7.2 with 1N NaOH.

(c) *Dye solution.*—Lanaperl fast pink R dye (American Hoechst Corp., Charlotte, NC), stock, 1% aqueous, 0.22 μm Millipore filter-sterilized.

(d) *Merthiolate solution.*—Thimerosal, NF powder (Elanco-Eli Lilly Co., Indianapolis, IN), stock, 1% aqueous.

(e) *Adjuvant.*—Freund's complete and incomplete adjuvants. Nos. 0638-60 and 0639-60 (Difco).

(f) *Normal bovine serum.*—Adult, product No. 14-400B (M.A. Bioproducts, Walkersville, MD).

(g) *Bovine serum albumin solution.*—Bovine serum albumin, No. A-4503 (Sigma Chemical Co., St. Louis, MO.), 5 mg/mL phosphate-buffered saline (b), 0.22 μm Millipore filter-sterilized.

Agar-Gel Immunodiffusion ORBIT Plate Preparation

Fill petri dishes (a) with 4 mL level, bubble-free immunodiffusion agar prepared in the following manner: Make a 1% concentration of purified agar (a) in pH 7.2 phosphate-buffered saline (b) and heat until agar is totally in solution and clear. Filter hot agar solution under vacuum through single layer of filtering cloth (b) in Büchner funnel on side-arm flask, and autoclave at standard conditions. After sterilization, aseptically add sufficient quantity of dye solution (c) to liquid agar and mix to effect final 1:60 000 concentration of dye. Cool agar solution to 60°C in water bath, add sufficient quantity of merthiolate solution (d), and mix to effect a final merthiolate concentration of 1:10 000. Dispense agar directly into plates or let harden in stock quantities and store at room temperature until remelted for future use. Keep prepared plates in sealed double plastic bags at 4°C until needed.

Antibovine Serum Preparation

Prepare specific antiserum against bovine species by immunization of New Zealand albino rabbits and sheep or goats with bovine immunogen preparations. Immunize rabbits by intramuscular primary injection of 5 mL Proom's (13) alum-precipitated bovine serum immunogen into each hind leg (10 mL total). Give 5 mL booster injection of same immunogen into one leg after 21 days, and make trial bleedings 7 days later. Separate sera from clotted blood, centrifuge, and freeze-dry onto paper discs as described for *Preparation of Stabi-*

lized Reagent Paper Discs. Make preliminary evaluation of sera suitability to intensity and specificity of immunoprecipitin reaction against homologous and heterologous tissue fluid-impregnated paper discs (both dried and nondried for homologous) within designed parameters of ORBIT procedure. Administer subsequent immunogen boosters, if necessary, in same manner and volume described at 3 week intervals, followed by appropriate trial bleedings and assays on sera to monitor suitability of antibody response.

Immunize goats or sheep by intramuscular injection into each of the two, rear, hind legs, biceps femoris muscles of 1 mL of a 1:1 emulsified mixture of Freund's complete adjuvant (e) and either normal bovine serum (f) or 5 mg bovine serum albumin (g) as the primary injection (2 mL total). Give subsequent booster injections (usually two) at 30 day intervals in same manner and volume as for primary injection, except use Freund's incomplete adjuvant. Make trial bleedings at 7–10 days after each booster, obtain sera and assay for their suitability to ORBIT procedure as described for rabbit antiserum.

Bovine Reference Extract Preparation

Prepare bovine reference extract from fresh, authentic beef (serologically confirmed), lean, whole muscle tissue as follows: Place 20 g sample finely diced beef tissue and 60 mL 0.85% saline in 22.8 × 11.4 cm Whirl-pak polyethylene bag, and macerate 15 s in stomacher. Let bag and it contents remain undisturbed for 30 min at room temperature, remove fluid, and centrifuge 20 min at 32 000 × *g* in refrigerated centrifuge. Decant supernatant beef extract, filter through Whatman No. 42 paper and centrifuge again as previously described. Finally, filter-sterilize supernatant beef extract through 0.22 μm filter into sterile containers, and store at 4°C until used to charge paper discs.

Preparation of Stabilized Reagent Paper Discs

Prepare stable bovine reference antigen discs and antibovine antibody discs by impregnating blank paper discs, spread on surface of 3.17 mm thick plexiglass sheets, with 40 μL or of prepared bovine reference extract suitably reacting antibovine serum. Let paper discs absorb these reagents 20 min at room temperature and then freeze directly by placing them at −10°C for 90 min. Freeze-dry discs overnight, under vacuum, on plexiglass in lyophilizer. Harvest reagent discs from plexiglass sheets the following morning, place in labeled screw-cap vials and store at 4°C until used for ORBIT.

ORBIT Procedure

Remove prepared agar-gel immunodiffusion ORBIT plates from refrigerator and let equilibrate to room temperature. The 4 lettered circles should be readily visible through the agar. Using fine-pointed forceps, carefully place flat on agar surface of ORBIT plate one antibovine antibody disc such that A-lettered circle of template is completely and evenly covered by disc when viewed directly from above. In an identical manner, place one bovine reference antigen disc over B-lettered circle of same plate. Use previously thawed, raw, whole muscle tissue test samples, and, in an area free of fat and connective tissue, make a vertical slice with a sharp knife to create a single slit ca 38 mm deep. Use forceps to place one blank sample paper disc halfway into depth of slit, flat against one side of tissue. Gently squeeze slit together so that both sides of sample disc are in contact with meat tissue. Let disc remain in this position 10 s to absorb tissue fluids. Remove sample disc from tissue slit and place, as previously described, over one of S-lettered circles of ORBIT plate containing positioned reference antigen and antibody discs. Treat second test sample in identical fashion and place that sample disc over remaining unoccupied S-lettered circle of same plate. Tightly seal lids on plates and leave undisturbed 24 h at normal room temperature to let reagents diffuse through agar and react. Then, examine plates in white, indirect light sources against flat, black background (Hyperion viewer) for formation of characteristic immunoprecipitin line in agar area among 4 positioned paper discs, and identify samples as bovine or nonbovine.

Results

Standardization of ORBIT Components and Conditions

A specific immunoprecipitin line can be obtained between reagent paper discs prepared by lyophilization of 40 μL bovine extract and antibovine serum, when they are positioned on the 4 mL agar plate surface. Rabbit, sheep, and goat antibovine sera all reacted well within the described parameters of this system, considering the limited antibody response produced by the immunization procedure used. Suitable antisera, which produced specific, well defined, intense immunoprecipitin lines between antigen and antibody discs within this system, could usually be obtained after 1 or 2 immunogen booster injections into the primed animal. Bovine serum albumin immunogen was preferable because of more consistent production of suitable antisera and the subsequent demonstration that the albumin anti-albumin reaction was the major component responsible for species identification of tissue fluids. Sheep and goat antibovine sera were preferable over rabbit antisera because they lack cross reactivity with sheep antigens and a larger serum volume can be collected from an immune animal. Because of the known, natural serologic relationship of ovine and bovine species (14), rabbit antibovine sera can demonstrate undesirable cross reactivity with sheep antigens, necessitating specific absorption of these sera to render them suitable for diagnostic purposes.

The distance between the inner edges of the discs, when positioned relative to each other on the agar surface, had a significant influence in determining the intensity and position of the immunoprecipitin band between reagent paper discs. A distance of 5 mm between reagent discs was the most suitable for producing a balanced, well defined, specific immunoprecipitin line.

Once the proper distance for disc placement was determined, a template was designed for silk-screen printing, in reverse, on the outside bottom of the petri dish. When viewed from above the agar surface, the pattern printed on an ORBIT plate (Figure 1) serves as a convenient, durable template for standardized placement of the antibovine antibody, bovine reference antigen, and 2 sample test discs in their proper relationship on the agar surface.

The temperature effect on the development of the immunoprecipitin line between antigen and antibody discs on an ORBIT plate was determined by placing identical test plates 24 h at 4, 23, and 37°C. The plates at 23 and 37°C produced a comparable, well developed immunoprecipitin reaction in the proper position; the 4°C plate produced a less intense reaction, less suitable for normal testing purposes. Thereafter, the standard development conditions of 24 h at 23°C were adopted, which allowed some variations in "normal" room temperatures in different environments without adversely affecting the desired reaction. Because a tight-fitting lid was used, agar dehydration during the 24 h reaction period did not occur at any temperature, so no humidity chambers were needed.

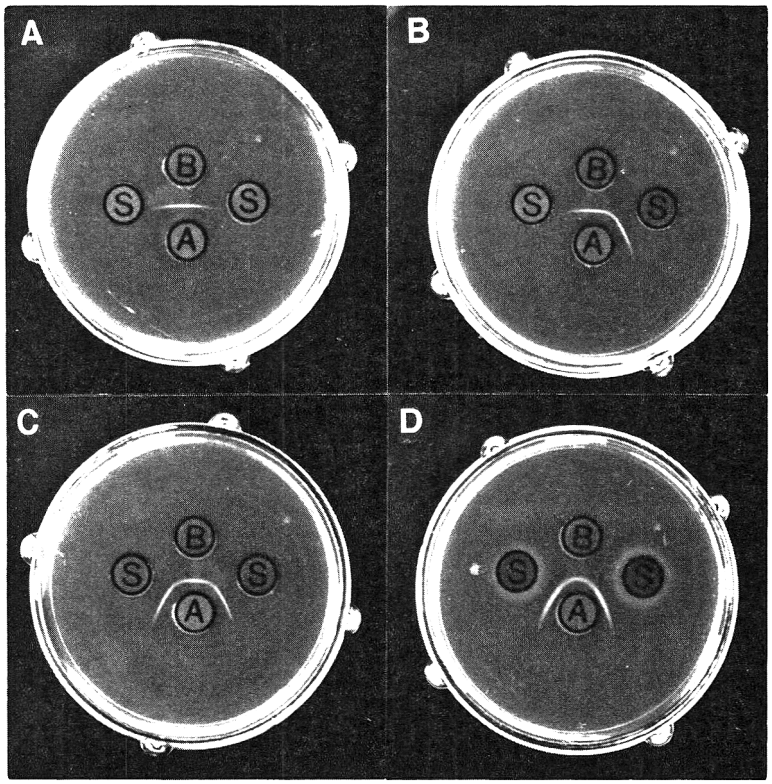

Figure 2. Typical ORBIT reaction patterns observed from routine meat sample analysis using sheep antibovine antibody reagent discs. Plate A, both samples nonbovine; plate B, sample on right bovine; plates C and D, both samples bovine.

Stability of Prepared Reagent Discs

To estimate prepared reagent disc stability under different time and storage conditions, we performed a shelf stability experiment. We divided a sufficient number of prepared antibovine antibody and bovine reference antigen discs between 2 screw-cap vials and stored them under 2 different test conditions. Periodically, antigen and antibody discs of the same age were placed on an ORBIT plate and allowed to react with each other, and the quality of the resulting immunoprecipitin band was compared with that produced by freshly prepared discs. Discs stored in the refrigerator (4°C) were considerably more stable than were those stored at room temperature (23°C); the period of maximum stability extended for at least 1 year. The diminished immunoprecipitin reaction observed at and beyond the 5 month period for discs stored at room temperature was found to be caused by deterioriation of the antibody disc. Antigen discs showed no evidence of instability when stored at room temperature for 1 year. These findings indicate the importance of the storage condition for prepared antibody reagent discs.

Sample Analysis

We performed a disc immersion time study to determine the length of time necessary for proper saturation of blank sample discs with tissue fluids, when placed in cut slits of meat test samples. Blank sample discs remained in cut slits of meat samples for 5, 10, 15, 30, 60, 120, 180, and 300 s, were then removed, placed opposite antibody discs on ORBIT plates, and allowed to react. Although all disc immersion times produced visible reactions, those left $\geq$ 1 min were considered too saturated because of the influence on the resulting immunoprecipitin line position and the presence of concentric rings of excessive proteinaceous material (nonspecific) around the sample discs. The standardized disc immersion time of 10 s selected for routine sample analysis produced highly suitable reactions with antibody discs and allowed proper disc saturation for the range of juiciness found among various meat samples. Figure 2 presents typical results observed on ORBIT plates for routine sample analysis under standardized conditions of the ORBIT procedure using sheep antibovine antibody reagent discs. Bovine samples produced an immunoprecipitin line of identity (complete fusion) with the bovine reference antigen line in the area among the discs; nonbovine related species produced no immunoprecipitin lines. The immunoprecipitin line of identity, resulting from the presence of 2 bovine test samples and the bovine reference antigen on an ORBIT plate, varied slightly in shape from a basic 3-sided figure to a curved arch type (Figure 2, plates C and D). Each type was occasionally skewed by a line formed between one of the test sample discs and the antibody disc. These variations were due to slight differences in the concentration of reacting antigen present in the saturated test sample discs and were not a problem in correctly interpreting the reaction and determining the nature of the test samples. The ORBIT procedure was designed and standardized to produce typical reactions (Figure 2) without spur formation or partial identity patterns that might complicate reaction interpretation for inexperienced field personnel.

We tested several samples of meat and poultry species to determine specificity of the ORBIT procedure for routine analyses. The related species of American bison (buffalo) and water buffalo (from Australia) were the only nonbovine species that gave positive bovine reactions (Table 1). Because these 2 species were previously shown by conventional immunodiffusion techniques to be very closely related to each other and to bovine, the demonstration of a positive bovine reaction for them by ORBIT was not surprising. This finding does not preclude the suitability of ORBIT as a basic screen test for imported/exported whole raw meat products in the

Table 1. Specificity of ORBIT procedure performed on meat and poultry samples of various species, using sheep antibovine antibody reagent discs

Species	ORBIT plate reaction[a]
Bovine	+
American bison (buffalo)	+
Water buffalo (from Australia)	+
Deer	–
Elk	–
Goat	–
Horse	–
Pig	–
Sheep	–
Red kangaroo *(M. rufus)*	–
Turkey	–
Chicken	–

[a] +, sample produced immunoprecipitin line of identity (complete fusion) with bovine reference line; –, sample produced no reaction.

Table 2. Results of laboratory and field trials of ORBIT performed on blind screen (unknown) meat and poultry samples, using sheep antibovine antibody reagent discs

Species[a]	No. of samples	ORBIT positive samples
Lab trial:		
Bovine	4	4
Bison	2	2
Deer	2	0
Elk	2	0
Horse	2	0
Sheep	2	0
Pig	2	0
Red kangaroo	2	0
Chicken	1	0
Turkey	1	0
Total	20	6
Field trial:[b]		
Bovine	45	45
Sheep	4	0
Horse	1	0
Total	50	45

[a] Identity of all samples was verified by Ouchterlony agar-gel, 2-dimensional, double-immunodiffusion technique (4) using whole antispecies sera and tissue extracts (except for bison and elk), and by using authentic reference tissue for laboratory trial samples and post examination of import inspection reports of the field trial samples.
[b] Tests conducted at Official Import Inspection Facility, Philadelphia, PA.

field. These buffalo species are rare, and are probably more expensive than beef when sold as labeled game meat. Therefore, they are unlikely to be used for adulteration or substitution. Isoelectric focusing could resolve any unusual case where an ORBIT positive sample is suspected of being buffalo, because this technique (11) can adequately differentiate these species.

ORBIT Performance Trials

To assess the reliability and practicality of using ORBIT in a kit form in the field, 2 trials were conducted using blind screen (unknown) samples: one in our laboratories under controlled testing conditions resembling those of a field situation, and the other as an actual field trial, at an Official Import Inspection Facility at Philadelphia, PA, on imported red meat samples collected by federal meat inspectors. The results (Table 2) show that all 49 bovine samples, of the 70 total samples examined, were correctly identified by positive ORBIT reactions. As expected, all other species of meat samples tested (except for the 2 bison samples) failed to give an ORBIT positive reaction. Thus, the ORBIT procedure was reliable, practical, and easy to use as a bovine screen test for field use. Less than 4 h was required by 2 individuals to complete the thawing, labeling, sampling, and plate setup for 50 samples at the inspection facility.

Discussion

In this report, we have presented a unique application of the agar-gel immunodiffusion technique using stabilized reagent components for the successful development of a bovine species serological screen test, which is highly suitable, practical, reliable, and economical for field use, such as at import inspection stations. The application of this test, involving primarily raw bovine products, for national and international meat inspection programs of the United States and elsewhere is expected to result in substantial cost reductions per sample analysis. By congressional mandate and Food Safety and Inspection Service Requirements, all nations exporting meat to the United States were required to have equivalent meat and poultry inspection systems and laboratory services in place by January 1984, so that imported products meet the domestic standards, especially for species verification. We anticipate that ORBIT will provide a major tool for these exporting nations to meet, in part, this equivalency requirement in an economically feasible manner. The cost reduction of sample analysis is due to the relatively low cost of producing each ORBIT unit, reduced need for sample shipment, fewer time delays, and elimination of disadvantages associated with other laboratory testing procedures. By using ORBIT as a screen test, the use of conventional serological (2–5) or electrophoretic (10, 11) species identification techniques will now be necessary only for those samples which give an ORBIT negative reaction.

A suitably reacting antiserum was crucial to the successful development and application of ORBIT, as is the case in most serological procedures. The preparation and use of sheep antibovine serum to provide the best specificity and balance required in ORBIT is especially important to assure no cross reactivity with ovine species antigens. Ovine meat is another major export product, so the use of this antiserum in ORBIT is important to prevent the possibility of obtaining false positive reactions and, therefore, improper species verification between bovine and ovine meat products. Although we chose to prepare our own antisera for the development of ORBIT, others who wish to establish this test system are not necessarily required to do so. During this investigation, we examined several commercially available preparations of rabbit, goat, and sheep antibovine sera, and many were found to be satisfactory for use in the ORBIT procedure. Some commercial antisera cross reacted with certain heterologous species. This is most likely a result of the tendency of manufacturers to make their antisera as strong as possible by hyperimmunizing primed animals, thereby reducing specificity to increase sensitivity, as previously reported by Tizard et al. (14). It is still possible, however, to render these antisera usable in the ORBIT procedure by making appropriate dilutions or absorbing them with the heterologous, cross-reacting antigens. The use of commercial antisera for implementing ORBIT where large scale testing programs are involved, would probably cost slightly more. Any antisera chosen would obviously need to be carefully examined and standardized within the parameters described in this report.

The finding that the close relationship of bison and water buffalo species to bovine produces a positive ORBIT reaction, although undesirable, is not considered a major problem in using this test, considering its intended application and the rare occurrences of these species.

Attempts to improve on this characteristic would likely involve the production of more specific antibovine serum, perhaps by absorption of existing antisera with bison and/or

water buffalo serum antigens. This does not appear to be promising, because of the intense cross reactions observed. Serum absorptions might produce a serum with a homologous bovine reaction that is too weak for use. An alternative approach to more specific antiserum production might be the development of monoclonal antibodies. The need or intended application of the test does not justify the effort and increased cost of the 2 approaches.

Although our basic system is based on the well known principle of immunodiffusion and the use of filter paper to convey immunological reagents, pioneered by Elek (12), there do not appear to be any reports of a similar application of this technique for the verification of meat species in large scale testing programs. The system we have presented is considered to have significant potential for further development, refinement, or application. It would appear probable that other ORBIT-like systems could be developed in a similar fashion for verification of other animal species if the need for large scale testing of such species should become necessary. With the use of a larger plate or container, it would also seem possible to produce a system whereby more than one species could be verified for one or several meat samples at the same time by using appropriate antispecies sera reagent discs. The basic ORBIT system also appears to be suitable for commercialization into stable test kits that contain all the necessary components and supplies so that they are readily available for species verification in large or small scale meat inspection programs.

Acknowledgments

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DRUG RESIDUES IN ANIMAL TISSUES

Gas Chromatographic Determination of Clopidol in Chicken Tissues

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A method has been developed for the determination of clopidol residues in chicken tissues. After extraction and cleanup, clopidol is esterified in a 2-phase system to clopidol propionate, which is determined by gas chromatography. The 2-phase system includes, in addition to the clopidol dissolved in methanol, aqueous borax solution, hexane, propionic anhydride, and pyridine. Use of these reagents precludes the use of explosive or carcinogenic chemicals in the derivatization step, and the method is therefore suitable for routine laboratory analysis. Levels of 0.5 ppb clopidol in tissue can be determined.

The coccidiostat clopidol (3,5-dichloro-2,6-dimethyl-4-pyridinol) is a widely used additive in poultry feed at levels of 100–200 mg/kg (1). Several previously described assays of clopidol in feed have a sensitivity range of 10–300 ppm (2, 3). Analysis of clopidol for the control of drug residues in poultry tissue requires a method with a substantially lower detection limit. Bjerke and Herman (4) and Suzuki et al. (5) have described a gas chromatographic (GC) method for the determination of traces of clopidol in biological tissues, and the latter authors presented an improved cleanup procedure. The final determination in these procedures is based on methylation of clopidol with diazomethane before GC analysis. However, diazomethane is a powerful carcinogen (6) and is also potentially explosive, which makes the method unsuitable for routine laboratory analysis. For this reason, a new GC method was developed which uses no hazardous reagents. In this method, the clopidol is esterified by reaction with propionic anhydride in alkaline solution (7, 8). The method includes a faster derivatization step compared with the former analytical procedures, and the determination limit is 0.5 ppb in the tissue samples.

METHOD

Apparatus

(a) *Cutter.*—Moulinette (Moulinex, France).

(b) *Homogenizer.*—MSE equipped with a 100 mL glass blender jar (Measuring & Scientific Equipment Ltd, UK).

(c) *Gas chromatograph.*—Varian Model 3700 equipped with ^{63}Ni electron capture detector (Varian) and glass chromatographic column (290 cm × 0.18 cm id) packed with 3% OV-17 on 80–100 mesh Chromosorb W A/W DMCS.

(d) *GC operating conditions.*—Injection port temperature 220°C; detector temperature 280°C. Column temperature programming: 130°C for 15 min, maximum temperature increase to 200°C for 4 min, to remove impurity peaks. Nitrogen carrier gas flow 35 mL/min.

(e) *Liquid chromatographic columns.*—2 cm (id) × 10 cm glass columns plugged with fritted disk of porosity grade 2, with Teflon stop cock, and equipped with 100 mL funnel top (Werner-Glas, Stockholm, Sweden). *(1) Aluminum oxide column.*—Pour 15 g aluminum oxide into column containing methanol and drain solvent to top of column. *(2) Anion-exchange column.*—Pour methanol slurry of QAE exchange resin in acetate form into column to bed height of 3 cm after settling, and drain solvent to top of column.

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(f) *Centrifuge* —Labofuge I (Heraeus, West Germany).

(g) *Buchner filters.*—*(1)* Porosity grade 2, disk diameter 6.5 cm (Pyrex, France). *(2)* Porosity grade 2, disk diameter 13 cm (Jenaer Glaswerk Scott & Gen., Mainz, West Germany).

(h) *Screw-cap tubes.*—10 and 30 mL with Teflon-lined plastic screw caps (Sovirel, France).

Reagents

(a) *Extraction solvents.*—Methanol GR (Riedel-De Haën, West Germany). Acetic acid, glacial, analytical reagent (BDH Chemicals Ltd, UK).

(b) *Derivatization reagents and solvents.*—Disodium tetraborate anhydrous GR (Merck, West Germany). Recrystallize the salt from hot solution before preparing 0.1M aqueous solution. *n*-Hexane for spectroscopy (Merck). Propionic anhydride Puriss (highest purity) (Fluka AG, West Germany). Distill the anhydride before use. Pyridine for spectroscopy (Merck).

(c) *Aluminum oxide.*—90 active basic, 70–230 mesh (Merck). Wash aluminum oxide several times with methanol and store in same solvent.

(d) *Anion-exchange resin.*—*(1)* QAE-Sephadex A-25 chloride form (Pharmacia Fine Chemicals, Sweden). Convert anion exchanger to acetate form in following manner: Let 100 g QAE swell in 10% aqueous sodium acetate solution for 2 h with occasional stirring. Pour slurry into Buchner filter (id 13 cm) and wash with 500 mL portions of 10% sodium acetate. Check for traces of chloride ions in the washing solution by adding a few drops of silver nitrate solution (acetone saturated with silver nitrate). If AgCl precipitate appears, rinse further with sodium acetate solution, but if solution becomes slightly cloudy, rinse with water until gel is chloride-free. Wash resin with 2 bed volumes of each of following solvents: methanol, 0.25% acetic acid in methanol and, finally, methanol. Store anion exchanger in methanol. *(2)* Anion exchange resin AG 1-X8, 100–200 mesh, acetate form, analytical grade (Bio-Rad Laboratories, Richmond, CA).

(e) *Clopidol standard solutions.*—Prepare stock solution by dissolving 10.0 mg clopidol analytical standard (Dow Chemical Co., Midland, MI) in 100 mL methanol (100 μg/mL). Dilute an aliquot of stock solution 1:100 with methanol to give an intermediate solution. Prepare working solutions ranging from 0.0025 to 0.10 μg/mL from this intermediate solution.

(f) *Filter aid.*—Hyflo Super Cel (Johns-Manville Products Corp., Denver, CO).

Extraction and Cleanup

The extraction and cleanup procedure is a slight modification of that described by Suzuki et al. (5).

Mince tissue sample with Moulinette cutter and transfer 20 g sample to an MSE homogenizer equipped with a 100 mL blender jar. Add 50 mL methanol and 3 g filter aid for muscle samples or 12 g for liver samples. Homogenize 3 min. Filter homogenate with suction through Buchner filter (internal diameter 6.5 cm) containing bed of 2 g filter aid, and collect

Table 1. Recovery of clopidol as function of acetic acid concentration in acetic acid methanol eluant, using 2 acetate form ion exchangers[a]

Concn, %, acetic acid in methanol (v/v)	Rec., %
AG 1-X8	
0.10	30
0.20	50
0.60	84
1.00	73
QAE-Sephadex	
0.10	78
0.15	89
0.20	95
0.30	81

[a]Conditions as describec in text. Anion exchange columns were of equal capacity. Clopidol concentration corresponded to tissue concentration of 20 ppb.

Table 2. Recovery of clopidol from spiked tissue samples, using 2 acetate form ion exchangers[a]

Tissue	Clopidol added, ppb	Rec., % Mean[b]	Std dev.
QAE-Sephadex			
Muscle	0.5	65	13.4
	2	79	3.1
	10	78	5.0
	20	76	6.3
	200	76	
	500	80	
	1000	86	
Liver	2	86	
	10	92	
	20	102	
	200	93	
	500	95	
	1000	93	
AG 1-X8			
Muscle	2	78	
	10	68	
	20	70	
Liver	2	40	
	10	56	
	20	50	

[a]Clopidol was eluted from QAE-Sephadex anion exchanger with 20 mL 0.2% acetic acid in methanol, and from AG 1-X8 with 20 mL 0.6% acetic acid in methanol.
[b]Each value is the average of 2 samples, except first 4 values in table are the average of 5 samples each.

filtrate in a 100 mL volumetric flask. Wash jar and filter cake with four 15 mL portions of methanol; adjust volume to 100 mL. Pipet a 50.0 mL aliquot onto alumina column, which is placed just above anion exchange column, and wash with 20 mL methanol. Discard eluate. Remove alumina column and wash anion exchanger with 20 mL 0.005% acetic acid–methanol. Elute clopidol with 20 mL 0.2% acetic acid–methanol from QAE anion exchanger into a 30 mL test tube with Teflon-lined screw cap. If AG 1-X8 anion exchanger is used, elute clopidol with 20 mL 0.6% acetic acid–methanol. Evaporate eluate to dryness on a water bath at 60°C under stream of nitrogen. Dissolve residue in 2.0 mL methanol.

Esterification and Determination

Transfer 1.0 mL aliquot to 10 mL test tube with Teflon-lined screw cap, containing 4.0 mL 0.1M aqueous borax solution. Add 1.0 mL hexane, 25 μL pyridine, and, finally, 50 μL propionic anhydride. Shake the 2-phase system and centrifuge at 2000 × g for 1 min. Transfer hexane layer to another test tube. Inject 6 μL hexane phase into chromatograph. Measure peak height and calculate concentration of clopidol by comparison with standard curve. Figure 1 shows a chromatogram of clopidol-fortified muscle tissue.

If many samples are to be analyzed, prepare a reagent mixture of hexane, pyridine, and propionic anhydride in advance.

Results and Discussion

In the cleanup procedure, we found that use of QAE-Sephadex anion exchanger, which is comparable to AG 1-X8, gave a somewhat less contaminated solution. The loss of clopidol when the AG 1-X8 resin was used was about 10% higher than that with the QAE exchanger for pure standard solutions (Table 1). The same phenomenon was observed when fortified muscle tissue samples were analyzed. Clopidol recovery from fortified liver samples was twice as high with the QAE exchanger (Table 2). Samples were spiked by adding clopidol standard solution to 20.0 g minced clopidol-free tissue. The total volume of methanol added was adjusted to 50 mL. The analysis was then carried out as described. The recovery would increase with a larger volume of eluant. However, a contaminated eluant could also increase the risk of interference in the final step of the determination, because the cleanup procedure includes an evaporation step.

Both acetic and propionic anhydrides were tested as derivatization reagents. Unfortunately, some impurities in the solvents gave a small peak in the chromatogram when acetic anhydride was used. This peak overlapped the clopidol acetate peak. The response of the electron capture detector was twice as high for clopidol propionate. For these reasons, we chose propionic anhydride as the derivatization reagent, although the subsequent chromatography required more time. Attempts to use halogenated reagents, such as monochloroacetic anhydride and trifluoroacetic anhydride, were unsuccessful. In the final step of the cleanup procedure, the sample is evaporated to dryness and then dissolved in a small volume of methanol. An aliquot of this methanol solution is taken for derivatization. It is advantageous if this methanol volume is small compared with the volume of 0.1M borax, because the yield of this step decreases with an increasing amount of methanol. However, it may be difficult to quantitatively dissolve the sample which is distributed over a large area in a 30 mL tube in a very small volume. The alternative that we chose was to dissolve the sample in 2 mL methanol and then take a 1 mL aliquot for further analysis.

A high concentration of propionic anhydride in the derivatization mixture resulted in an increased detector response and a high background level in the chromatogram. The same result was obtained if the ratio of pyridine to propionic anhydride was kept constant during the procedure. An increased concentration of pyridine produced no change in the peak height, only increased tailing.

GC-MS (LKB 2091 gas chromatograph-mass spectrometer) was used to confirm the clopidol propionate peak. Two clopidol standard solutions containing 1 and 20 μg clopidol/mL were derivatized before GC-MS analysis. Figure 2 shows the mass spectrum for the solution with the higher concentration. The main peak in the spectrum at m/z 191 corresponds to a loss of 56 mass units from the clopidol proprionate molecule, which is typical for propionates. The molecular ion at m/z 247 shows an isotopic cluster characteristic of a molecule containing 2 chlorine atoms.

Lindane (γ-hexachlorocyclohexane) was tested as an internal standard, but was rejected because its retention time was

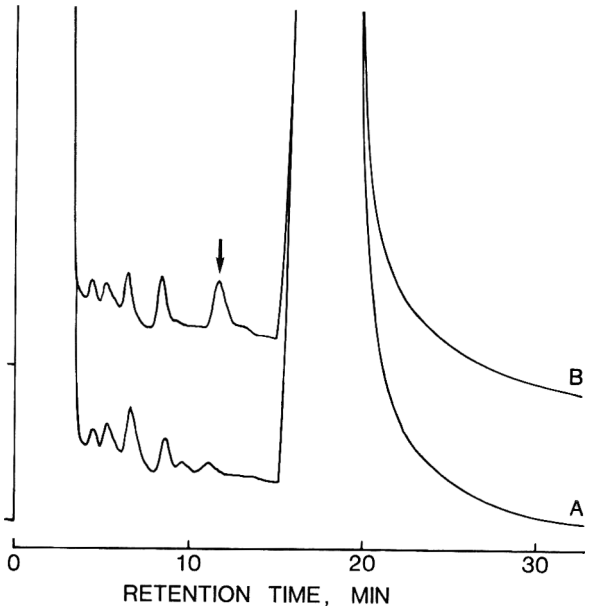

Figure 1. GC chromatogram of (a) muscle tissue and (b) muscle tissue fortified with 2 ppb clopidol.

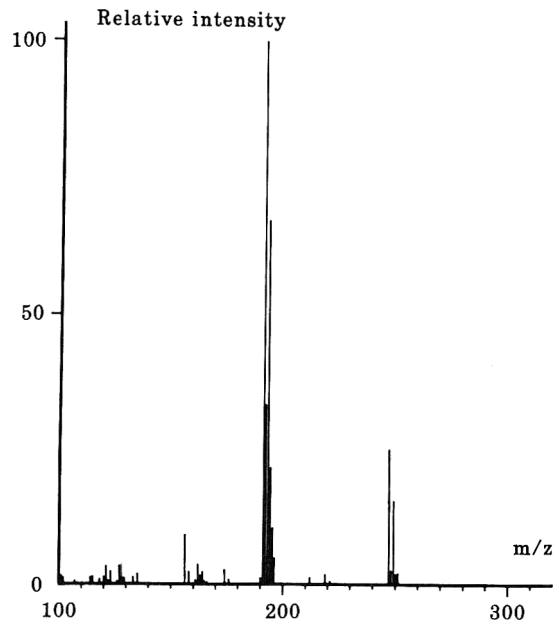

Figure 2. Mass spectrum of clopidol propionate peak.

long compared with that for clopidol propionate, and the coefficient of variation was not better for the peak ratios compared with the peak height for clopidol propionate. The repeatability of the injected sample volume was ≤1%.

A linear relationship between peak height and concentration was found in the tested range, 0.0025–0.10 μg/mL, which corresponds to a tissue concentration of 0.5–20 ppb.

The limit of detection, c_L, was calculated in accordance with the recommendations of the International Union of Pure and Applied Chemistry (IUPAC) (9, 10). In defining c_L, IUPAC states that $x_L = \bar{x}_B + k \times s_B$, where x_L is the smallest detectable signal, $\bar{x}_B$ is the mean value of the blank response with its standard deviation s_B, and k is the numerical factor chosen in accordance with the desired confidence level. The relation between c_L and x_L is given by the expression $c_L = (x_L - \bar{x}_B)/m$, where m is the analytical sensitivity. The use of k = 3 allows a confidence level of 99.9%. Determination of 11 different blank samples resulted in $\bar{x}_B = 3.0$ with $s_B = 0.8$. This gives a limit of detection, $c_{L(k=3)}$, of 0.1 ppb. Variation of m, which was found to be 4%, has not been taken into consideration. The ACS Subcommittee on Environmental Analytical Chemistry has proposed a stricter statistical criterion for separating the blank signal from the true signal (10, 11). The criterion is called the limit of quantification (LOQ), which is defined as 10 s_B away from $\bar{x}_B$. With the above values of $\bar{x}_B$ and s_B and considering that the recovery is only 65% in this concentration range, the calculated limit of quantitation, c_{LOQ}, is 0.5 ppb.

From the data in Table 2 it can be calculated that the relative standard deviation is 21% for a muscle sample spiked with 0.5 ppb clopidol, which is more than twice as high as that for other tissue samples spiked with higher concentrations of clopidol. However, both the c_{LOQ} value and the drastic increase of the standard deviation for samples spiked with 0.5 ppb clopidol indicate that 0.5 ppb is the limit of determination.

Thus, high sensitivity and rapidity, as well as good reproducibility without the use of potentially hazardous chemicals, render the method suitable for routine analysis of clopidol residues in chicken tissue.

Acknowledgments

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Liquid Chromatographic Determination of Carbadox, Desoxycarbadox, and Nitrofurazones in Pork Tissues

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A liquid chromatographic (LC) method has been developed for the determination of carbadox, desoxycarbadox, and nitrofurazones in the 10–40 ppb range in pork muscle, liver, and kidney tissues. Tissues were homogenized in absolute ethanol, and the homogenates were treated with metaphosphoric acid and reduced in volume by rotovaporization. Hexane was added to the concentrates, which were then centrifuged to remove fat. After addition of KH_2PO_4 to the aqueous phase and extraction with ethyl acetate, the extracts were passed through alumina columns before analysis by reverse phase LC. Overall average recoveries (10–40 ppb range) for carbadox and desoxycarbadox from spiked tissues were 53% ± 13.6 and 61% ± 7.2, respectively; overall average recoveries for nitrofurazone and furazolidone were 43% ± 7.3 and 77% ± 10.9, respectively. Before these optimum determinations, degradation by even minimal incandescent light was found to reduce recovery especially of desoxycarbadox. The results of this photochemical degradation are reported and briefly discussed.

Since Kornegay's discovery in 1968 of the value of carbadox for the prevention and treatment of swine dysentery (1), this drug has been used in a number of countries under the brand names Mecadox® and Fortigo® (Pfizer) to control certain swine enteric diseases and to promote swine growth (2). A withdrawal period of 10 weeks is required by the U.S. Food and Drug Administration (FDA) before slaughtering pigs (up to 75 lb) fed carbadox-treated feeds. A 35-day withdrawal period with the same 75 lb weight limit is allowed by Canada Agriculture. These regulations exist because of the carcinogenic properties of carbadox and especially of its metabolite, desoxycarbadox (3). Although carbadox has also been shown to be a strong mutagen in both mice and *Klebsiella pneumoniae* (4), it appears to have a high safety factor (9000) in "relay toxicity" trials conducted on dogs fed pork from animals that were raised on carbadox-treated feed (5). At present, the *U.S. Code of Federal Regulations* (6) specifies a zero tolerance for residues of this drug in the uncooked, edible tissues of swine.

Although the *Federal Register* (7) describes a general gas chromatographic (GC) method for determining carbadox and its metabolites as quinoxaline-2-carboxylic acid, no methodology has been developed for the specific identification and quantitation of carbadox and desoxycarbadox in animal tissues[1] consumed for food; however, liquid chromatographic (LC) methods have been reported for carbadox in animal feeds (8, 9). The specificity of the LC methodology was developed as an advance over the older colorimetric assay for carbadox in swine feeds (10), which had evolved to a high level of improvement and collaborative study (11, 12). In the present *Federal Register* method (7) for enforcing the zero tolerance level for residues of carbadox in uncooked, edible tissues of swine, the specific chemical identity of carbadox and any of its metabolites is necessarily destroyed because all entities are hydrolyzed to the common entity, quinoxaline-2-carboxylic acid, which is monitored by GC after derivatization to its propyl ester. The present LC method was developed to permit the specific identification and assay of carbadox and its degradation products in swine tissues. The method may also be used to screen samples for nitrofurazone and furazolidone.

Experimental

Clean all glassware with Decon 75 (BDH Chemicals). Use only glass-distilled (or equivalent) solvents and reagant grade chemicals. Acetonitrile was LC grade with 190 nm UV cutoff. Note particularly that samples and standards must be protected from both UV light and moderate incandescent lighting. Caution: Desoxycarbadox is labeled as a carcinogenic compound.

Reagents

(a) *Standards.*—Carbadox and desoxycarbadox: Pfizer Canada Inc., Kirkland, Quebec, Canada. Nitrofurazone and furazolidone: Norwich-Eaton Pharmaceuticals, Norwich, NY 13815.

(b) *Standard solutions.*—Store in a cool, dark place when not in use. *(1) Stock solutions.*—1 mg/mL. Dissolve 10 mg of each standard in dimethylformamide (DMF) in separate 10 mL volumetric flasks, and dilute each to volume with DMF. *(2) Working solution 1.*—10 μg/mL of each standard. Combine 1.0 mL each stock solution and dilute to volume with mobile phase (acetonitrile–0.01M ammonium acetate–ethanol (25 + 70 + 5)). *(3) Working solution 2.*—1 μg/mL. Dilute 1.0 mL working solution 1 to 10 mL with mobile phase. Prepare working solution 2 fresh each day. Protect all solutions from sunlight and UV light.

(c) *Aluminum oxide.*—Baker Analyzed Reagent (80–200 mesh) suitable for chromatographic use. pH of 10% slurry at 25°C is 6.3.

Apparatus

(a) *Liquid chromatograph.*—Waters Associates Model ALC/GPC-204, equipped with U6K injector and M6000A solvent delivery system.

(b) *Detector.*—Perkin Elmer LC-75 variable wavelength set at 350 nm coupled to Hewlett-Packard recorder (Model 3385A) for recording retention times and responses measured as peak height.

(c) *Column.*—Brownlee RP-10A, reverse phase, C-8 (RP-8), 10 μm, 4.6 mm id × 25 cm with Brownlee RP-GU MPLC guard column with C-8 (RP-8) 10 μm packing. Operating conditions: mobile phase, acetonitrile–0.01M ammonium acetate–ethanol (25 + 70 + 5); flow rate, 1 mL/min; injection volume, 100 μL.

(d) *Blender.*—Waring, with stainless steel chamber and blades.

(e) *Chromatographic columns.*—Kontes, No. K-410100 Chromaflex, 6 × 200 mm with 50 mL reservoir volume (Kontes Glass Co., Vineland, NJ 08360).

(f) *Centrifuge.*—Refrigerated Sorval RC2-B with SS-34 head.

[1]Since release of this manuscript by the Bureau of Drug Research for publication, a GC-MS method for confirmatory identification of carbadox-related residues in swine liver was published by Lynch and Bartolucci, *J. Assoc. Off. Anal. Chem.* **65**, 66–70 (1982). However, their method is based on detection of the end degradation product, quinoxaline-2-carboxylic acid, as its methyl ester derivative, and therefore, it does not identify and distinguish specific carbadox residues.

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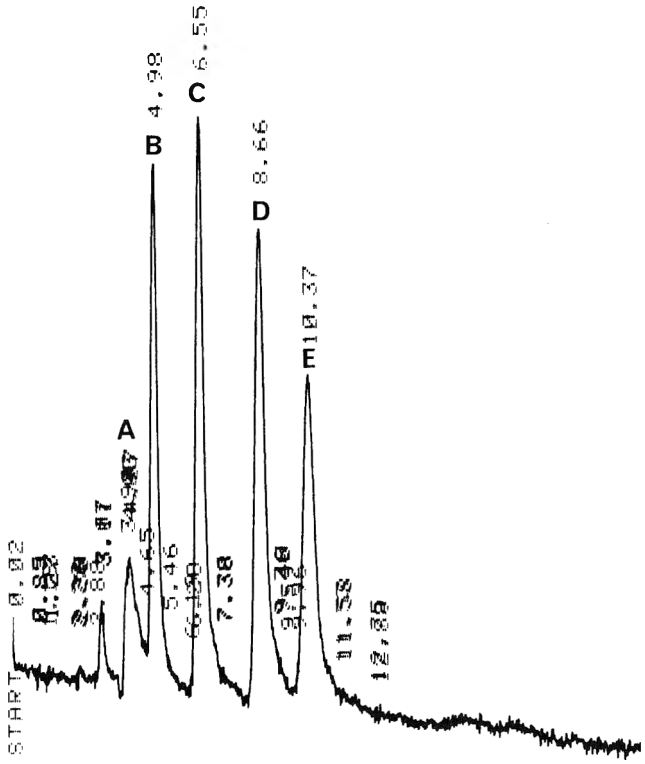

Figure 1. LC separation (20 μL injection) of mixed reference compounds (10 ng each); quinoxaline-2-carboxylic acid (A), carbadox (B), nitrofurazone (C), furazolidone (D), and desoxycarbadox (E) under optimized conditions and UV detection at 350 nm. Extra early eluting peak is a persistent background peak.

Standard Curve for Spiked Tissue

Add 20, 100, 200, 300, and 400 ng of *working solution 2* to 10 g tissue (2, 10, 20, 30, and 40 ppb). Determine recovery for each concentration by comparing peak heights with those for direct LC injections of 4, 20, 40, 60, and 80 μL *working solution 2* (2, 10, 20, 30, and 40 ng), respectively.

Preparation of Samples

Remove fat before cutting fresh tissues into small pieces (<1 cu. cm) with kitchen scissors. Pool and mix all material by stirring with spatula to make uniform sample. Weigh 10 g aliquots, wrap in Saran, and store composite specimens in a freezer.

When tissue specimens are needed for analysis, thaw the frozen specimens (10 s/10 g) in microwave oven. Store thawed tissue in refrigerator until processed.

Extraction and Cleanup

To prepare standard curves, use Hamilton syringes to add appropriate volumes of standard solutions of carbadox and desoxycarbadox (for 2, 10, 20, 30, or 40 ppb each drug) to 10 g tissue in blender. Homogenize specimens (spiked, blank, or assay tissue) 5 min in 100 mL absolute ethanol in the blender. Let the homogenate stand 5 min before filtering it through a layer of Celite 545 (10 g) applied to sintered glass (medium porosity, 350 mL) filter. Rinse blender with 100 mL absolute ethanol, and pass rinsings through filter into first filtrate.

Transfer combined filtrate to 1 L round-bottom flask, and add 25 mL 3.6% aqueous metaphosphoric acid to aid precipitation of foreign material. Reduce volume of mixture to ca 25 mL by rotary evaporation at 45°C. Do not let sample evaporate to dryness, or some residue will be lost. Transfer concentrate to 50 mL centrifuge tube. Rinse flask by swirling with 5 mL hexane, and then with ca 3 mL water, and add rinsings to centrifuge tube. Centrifuge 30 min at 1500 rpm (for SS-34 head = 27 000 × *g*) and 0°C. Remove and discard upper hexane layer. Rinse surface carefully with additional 5 mL hexane, and discard rinsings.

Transfer lower, aqueous layer to 250 mL separatory funnel without disturbing precipitate. Rinse centrifuge tube and any adhering material gently with two ca 3 mL portions of water, and add rinsings to separatory funnel. Add 10 mL 1M KH_2PO_4 solution to funnel to create salting-out effect, and add water to total volume of ca 100 mL. Extract mixture with three 50 mL portions of ethyl acetate (5-min extractions), and dry combined extract over 15 g granular, anhydrous Na_2SO_4.

Filter dried ethyl acetate extract through plug of glass wool into 500 mL round-bottom flask, and reduce extract to dryness by rotary evaporation at 45°C. Prolonged drying results in loss of (residue) sample. At this stage, residue may be stored overnight in same flask in a refrigerator if there is not enough time to complete analysis. For final sample cleanup, dissolve residue in 3 mL ethyl acetate, and transfer solution to small alumina column (previously prepared by slurrying 1 g Baker aluminum oxide with ca 20 mL ethyl acetate into 6 × 200 mm chromatographic column). Rinse flask with 2 mL ethyl acetate, and add rinsings to column. Some carbadox is eluted with ethyl acetate, so all ethyl acetate should be collected. Elute carbadox and desoxycarbadox residues into retained ethyl acetate with 20 mL of mixed ethanol–methanol–ethyl acetate (10 + 10 + 80); then reduce total volume (25 mL) to dryness in 100 mL pear-shape flask by rotary evaporation at 45°C.

Finally, reconstitute residue in 500 μL mobile phase (acetonitrile–buffer–ethanol (25 + 70 + 5, pH 6.8), and inject 100 μL aliquots onto LC column (Brownlee RP-8, 10 μm packing, 4.6 mm id × 25 cm). Maintain constant 1.0 mL/min flow of mobile phase through column, and monitor effluent at 350 nm by using flow-through cell.

Discussion

Optimization of LC and UV Detection

The selection of a reverse phase LC column for the determination of carbadox, desoxycarbadox, and quinoxaline-2-carboxylic acid was based on previous experience with LC analysis of nitrofuran drugs (13, 14). Of the various columns tried, a Brownlee RP-8 column was best not only for carbadox and its metabolites but also for nitrofurazone and furazolidone, thereby permitting simultaneous analysis for these drugs as a screening method in tissue. With this column, overall elution time was 2–10 min, with good resolution for the 5 compounds at a 1 mL/min flow rate. Separation (Figure 1) was optimum with the acetonitrile–buffer–ethanol (25 + 70 + 5) mobile phase adjusted to pH 6.8 with 0.1N ammonium hydroxide.

Carbadox and desoxycarbadox each presented a problem for dissolution in common solvents because they are virtually insoluble in water, methanol, ethanol, ethyl acetate, acetonitrile, hexane, and even corn oil. This problem was circumvented by preparing stock solutions of each drug (1 mg/mL) in dimethylformamide, which were then diluted with mobile phase. Other workers have used dimethyl sulfoxide (4, 9) to prepare carbadox stock solutions; one worker used ultrasonic dissolution in $CHCl_3$–methanol (3 + 1) (10).

Drugs were detected by a variable wavelength UV detector placed immediately downstream from the LC column. We had to accommodate a number of factors to obtain optimum

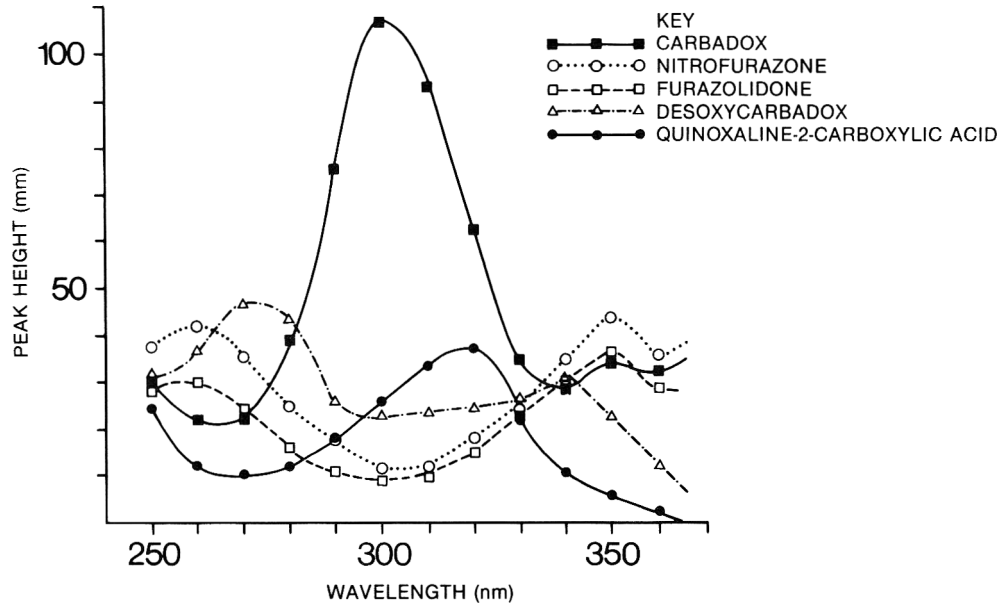

Figure 2. UV absorption of carbadox, desoxycarbadox, quinoxaline-2-carboxylic acid, nitrofurazone, and furazolidone under LC operating conditions. Compiled from 30 ng injections of each reference substance in acetonitrile–water–ethanol (25 + 70 + 5) mobile phase at 1.0 mL/min flow rate and attenuation setting × 6.

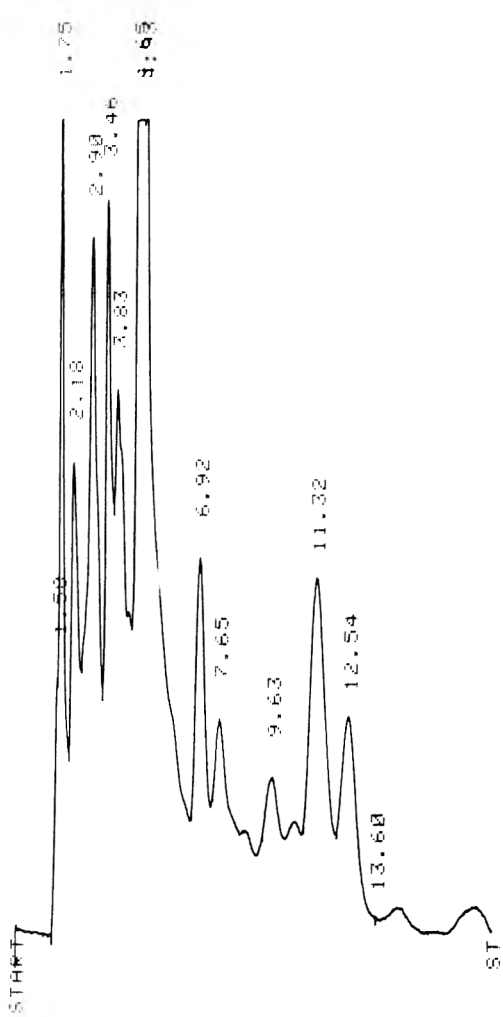

Figure 3. Example of high background interference from a pork muscle blank (100 μL injection equivalent to 2 g tissue) monitored at 290 nm after the described extraction and cleanup.

detection. From the UV absorption profiles that were manually determined for the 5 compounds (Figure 2), it was apparent that 350 nm would provide optimal sensitivity for simultaneous monitoring of all five. Unfortunately, the absorption of quinoxaline-2-carboxylic acid at 350 nm is not as great as that of the other drugs; however, its absorption at 350 nm is not much less than at 280 nm, a wavelength that offers roughly comparable absorption strength for carbadox and desoxycarbadox, but less response for the nitrofuran drugs. While the greatest detection sensitivity for carbadox would be obtained near 300 nm with adequate response for desoxycarbadox and relatively greater response for quinoxaline-2-carboxylic acid, absorption by the nitrofuran drugs is minimal. Maximum absorption for quinoxaline-2-carboxylic acid would be obtained near 320 nm, complemented with high absorption for carbadox, adequate absorption for desoxycarbadox, and reduced absorption for the nitrofuran drugs. The 350 nm wavelength was selected because there was very high background interference below 340 nm from tissue UV-absorbing components that were carried through the extraction and cleanup procedures (Figure 3).

Extraction and Cleanup of Tissues

The effect of different solvent combinations for extraction during homogenization was investigated to optimize dissolution and to minimize subsequent cleanup of the extracts. Tissue samples were homogenized with methanol–water (1 + 1), ethanol–water (1 + 1), 70% ethanol, 100% ethanol, and isopropyl alcohol. Of these, 100% ethanol gave the best recovery and cleanest separation. To reduce the fatty content of tissue at an earlier stage in the workup, homogenization was tried with ethyl acetate and with hexane followed by ethyl acetate extraction. There was no significant benefit from either of these procedures.

After tissue homogenization with 100% ethanol, it was beneficial to remove tissue solids by passing the homogenate through a Celite filter pad. We observed that precipitation with aqueous metaphosphoric acid reduced the number of ethanol-soluble UV-absorbing components in the homogenate. In these trials, we found that addition of neither 0.1M ammonium acetate nor solid KH_2PO_4 to the ethanolic homogenate was effective in promoting sample cleanup.

After concentrating the treated ethanolic homogenate by rotary evaporation, the concentrate and added hexane were centrifuged to precipitate solid matter and to partition fat into

Table 1. Data on calibration curves for reference compounds

	Carbadox			Desoxycarbadox			Nitrofurazone			Furazolidone		
ng	Peak ht[a]	Std dev.	CV	Peak ht	Std dev.	CV	Peak ht	Std dev.	CV	Peak ht	Std dev.	CV
2	18.6	0.9	4.9	10.8	0.9	7.9	20.7	0.8	4.0	17.8	0.9	4.9
10	84.4	2.1	2.5	49.7	4.8	9.6	95.0	4.7	5.0	80.7	4.9	6.1
20	159	1.5	1.0	97.8	5.0	5.1	182	3.3	1.8	156	4.1	2.6
30	225	1.7	0.8	142	6.7	4.7	259	4.4	1.7	225	5.4	2.4
40	296	1.6	0.5	194	3.1	1.6	340	1.9	0.5	298	1.8	0.6
	$y = 7.23x + 9.12$ $r = 0.999$			$y = 4.77x + 1.48$ $r = 0.999$			$y = 8.35x + 8.92$ $r = 0.999$			$y = 7.32x + 5.92$ $r = 0.999$		

[a]All peak heights normalized for attenuation setting 4, average of 18 injections using 2 dilutions of 3 solutions.

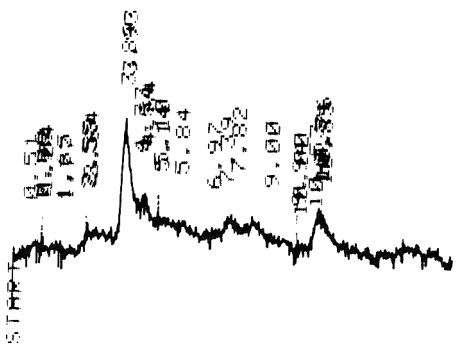

Figure 4. Typical LC chromatogram obtained from a pork kidney blank. 100 μL injection (equivalent to 2 g tissue) and UV detection at 350 nm.

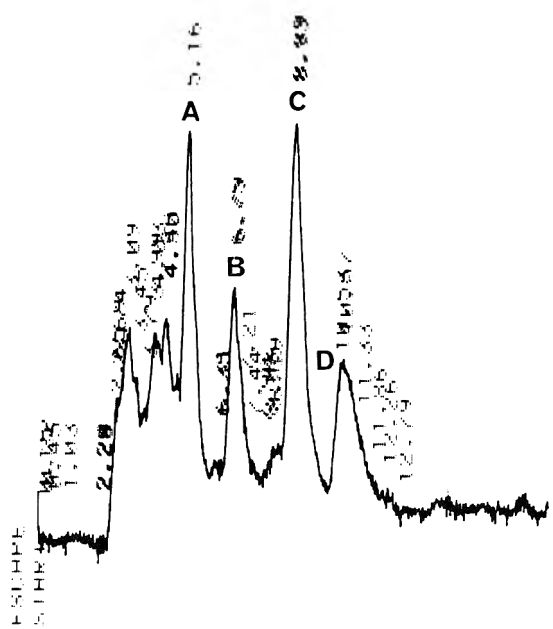

Figure 5. Typical LC chromatogram from pork kidney spiked with carbadox (A), nitrofurazone (B), furazolidone (C), and desoxycarbadox (D) (each 10 ng). 100 μL injection (equivalent to 2 g tissue) and UV detection at 350 nm.

the upper hexane layer. Fatty material was then carefully removed with the hexane from the centrifuge tube, and a second hexane wash was applied to the surface to remove any remaining fatty material.

Extraction of the concentrate was enhanced by the salting-out effect produced by the addition of 10 mL 1M KH_2PO_4 during transfer of the concentrate and water washings to a separatory funnel. The water-soluble components were left behind after ethyl acetate extraction. Whereas sodium chloride or hydrochloric acid has been used effectively at this stage for other drugs (13, 14), it is noteworthy that use of either of these reagents in this work resulted in reduced recovery of carbadox and desoxycarbadox.

For final cleanup of the tissue extract, we tried several column materials (silica, alumina, alumina plus carbon, Florisil, and combinations of Florisil and alumina). Only alumina was effective; 1 g provided optimum separation for the weights of packing tried (0.5, 1.0, 1.5, and 2.0 g). The effectiveness of various solvent mixtures for elution of each column packing was also investigated. For alumina, ethanol–methanol–ethyl acetate (10 + 10 + 80) was a more effective eluant than was ethanol–ethyl acetate (20 + 80), acetonitrile–ethyl acetate (20 + 80), or ethanol–ethyl acetate (30 + 70). After evaporation to dryness, the column eluate was reconstituted in 500 μL mobile phase, and a 100 μL aliquot was used for LC injection. Low recoveries sometimes occur because of the difficulty in dissolving the residue in mobile phase; however, warming the mixture on a 45°C water bath aids dissolution. Unfortunately, this operation also results in carrying more fatty material into the LC system, which tends to separate and collect in the guard column. Periodic reverse washing or replacement of the guard column obviates these problems. Kidney tissue, nevertheless, presents the greatest difficulty for analysis because of the nature of its fatty content.

Results

Table 1 presents data used to prepare the calibration curves for the reference compounds, carbadox, desoxycarbadox, nitrofurazone, and furazolidone.

Table 2 summarizes the results of assaying spiked tissues (muscle, kidney, and liver randomly purchased from grocery outlets) for carbadox, desoxycarbadox, nitrofurazone, and furazolidone by the LC method. Figures 4 and 5 show typical chromatograms for a pork kidney blank and pork kidney spiked at the 10 ng level, respectively.

The variability of assays of composite material, as evidenced by the tabulated CV values (Table 2), appears to be caused by problems inherent in extraction procedures and by difficulty in reconstituting the final residue in mobile phase as discussed earlier. Although the residues can be detected at 2 ppb, the high CV values and recoveries indicate that the method is not reliable at that level.

Furthermore, in assays of fresh liver samples obtained by autopsy from a pig fed carbadox-treated ration, we found that even minimal incandescent light can reduce assay levels and give results that vary in proportion to distance from the lamp and duration of exposure. We observed this degradative influence of minimal incandescent light after a complete assay of tissues (comparable to Table 2) resulted in overall average recoveries for carbadox and desoxycarbadox from spiked tissues (10–40 ppb) of 50% ± 8.8 and 54% ± 10.8, respectively, and correspondingly overall average recoveries of 40% ± 12.3 (nitrofurazone) and 75% ± 17.2 (furazolidone). As a

Table 2. Recovery of carbadox, desoxycarbadox, nitrofurazone, and furazolidone added to pork tissues[a]

	Carbadox				Desoxycarbadox				Nitrofurazone				Furazolidone			
ng	Peak ht	SD	CV	Rec., %	Peak ht	SD	CV	Rec., %	Peak ht	SD	CV	Rec., %	Peak ht	SD	CV	Rec., %
								Kidney								
2	10.5	1.8	16.9	56	10.5	2.4	23.3	97	9.4	3.9	41.9	45	14.3	1.0	7.3	80
10	32.6	3.3	10.2	39	27.6	5.3	19.4	55	29.8	5.1	17.1	31	57.7	4.2	7.3	71
20	73.6	10.9	14.8	46	51.3	9.6	18.7	52	65.6	9.5	14.5	36	114	9.7	8.5	73
30	116.8	14.9	12.8	52	69.8	14.2	20.0	49	115	44.7	38.9	44	170	18.0	10.6	76
40	160	32.6	20.4	54	118	22.8	19.3	61	139	36.6	26.4	41	241	34.5	14.3	81
								Liver								
2	21	4.8	22.8	112	10.6	2.9	27.3	98	7.3	1.4	19.3	35	25.8	1.5	5.7	145
10	62.7	8.5	11.7	74	32.3	3.0	9.3	65	33	3.6	10.8	35	73.9	14.6	19.8	92
20	113	18.1	16.0	71	57.7	11.8	20.5	59	64.3	10.0	15.5	35	128	12.4	9.7	82
30	158	20.3	12.8	70	86.9	8.1	9.3	61	121	13.2	10.9	47	192	31.4	16.3	86
40	193	41.7	21.6	65	128	21.9	17.0	66	164	24.0	14.6	48	281	43.1	15.3	94
								Muscle								
2	12.6	1.5	12.0	68	8.1	1.7	20.1	75	8.8	1.3	14.8	42	17.4	4.4	25.2	98
10	32.9	3.1	10.1	39	35.3	2.0	5.6	71	43.8	6.8	15.5	46	47.6	6.4	13.4	59
20	70.6	12.4	17.6	44	63.6	7.4	11.6	65	91.6	16.3	10.4	50	95.6	9.0	9.4	61
30	92.2	16.7	18.1	41	103	17.1	16.6	73	138	14.9	10.8	53	170	7.0	4.1	76
40	118	17.6	14.9	40	117	25.4	21.8	60	174	21.6	12.5	51	211	15.3	7.3	71

[a]Data represent an average of 8 injections (minimum) based on peak heights normalized for attenuation setting 4. A composite sample was prepared for each tissue; from each of these composites, 4 aliquots were spiked to each of the indicated levels, and at least 2 injections were made from each aliquot.

Table 3. Effect of lamp distance on level of desoxycarbadox residue in pig liver

Sample[a]	Distance to lamp, cm	Av. peak ht, mm	Desoxycarbadox, ppb
a	65	185	38
b	75	247	51
c	85	312	65

[a]Each sample (10 g) was a composite of finely cut liver tissue obtained by autopsy from a pig fed carbadox-treated ration [50 g/ton (2000 lb) (0.0055%) of finished feed] for 3 weeks and slaughtered at zero withdrawal time.

result of this concern, we repeated the extensive assay work on new tissue samples and obtained the improved results summarized in Table 2. Overall average recoveries (10–40 ppb range) for carbadox and desoxycarbadox from spiked tissues were 53% ± 13.6 and 61% ± 7.2, respectively; overall average recoveries for nitrofurazone and furazolidone were 43% ± 7.3 and 77% ± 10.9, respectively.

As mentioned earlier, we assayed fresh liver samples from a pig fed carbadox-treated ration; the results of in-house trials involving carbadox-fed pigs will be published later. We noted in that work that there was a progressive reduction in desoxycarbadox content corresponding to the order of workup position of 3 identical tissue samples within the fumehood (exposed for the same length of time) in respect to a 60-watt incandescent lamp (Table 3). Although the samples were protected from exposure to direct light by a reflector attached to the lamp to direct illumination into a back corner of the hood (and no overhead laboratory illumination as usual for any of the assays), sufficient indirect illumination reached the samples during their limited preparation (~2 h) within the hood to cause proportionate degradation of desoxycarbadox. When this assay was repeated in the absence of any light in the fumehood, using fresh liver samples from the same batch (same animal), uniform assay results for desoxycarbadox were obtained. This observation and its experimental corroboration demonstrates the great sensitivity of desoxycarbadox in solution to light of even the weakest UV source. Although stringent precautions had been taken to conduct all tissue assays in near darkness, these results showed that no illumination could be tolerated during fumehood operations, a conclusion supported by the more consistent results and more acceptable CV values obtained when all tissue samples were re-assayed in virtual darkness.

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MYCOTOXINS

Mycotoxins in Grain Dust: Method for Analysis of Aflatoxins, Ochratoxin A, Zearalenone, Vomitoxin, and Secalonic Acid D

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A multimycotoxin method is presented to quantitate aflatoxins, ochratoxin A, zearalenone, secalonic acid D, and vomitoxin in grain dust. Dust spiked with these mycotoxins was extracted sequentially with methylene chloride followed by acetonitrile–water (86 + 14). Vomitoxin was recovered in the latter extract and all other mycotoxins were recovered in the methylene chloride. Aflatoxins and ochratoxin were quantitated by fluorescence measurement on silica thin layer chromatographic plates. The other mycotoxins were quantitated after cleanup by reverse phase liquid chromatography and ultraviolet detection. Recoveries from dust spiked in the parts per billion (ng/g) range were approximately 80% (SD = 15–29%) for all mycotoxins. Minimum detectable amounts ranged from less than 0.5 ng/g for aflatoxins to 20 ng/g for zearalenone.

Several multimycotoxin methods have been developed previously to simultaneously extract a number of mycotoxins from corn (1–9). Grains and oilseeds handled by elevators in the New Orleans area are mainly soybean, corn, and wheat. Dust generated at these storage and shipping points reflect this composition. Because of this multiplicity of sources of grain dust, methods designed for a single type of grain may require modification. The method reported here was adapted from the official AOAC method for aflatoxins (10), a modification of the van Egmond procedure for ochratoxin A (11), and the procedure of Ware and Thorpe for zearalenone (12). Isolation and quantitation of secalonic acid D and vomitoxin were accomplished by modifications of previously described methods (13, 14).

All mycotoxins considered in this study are possible contaminants of corn (15–20), and some were reported as natural contaminants of wheat (21–26). As these grains are processed during shipping and storage, dust particles are formed. The dust particles may contain elevated levels of mycotoxins compared with the original grains because mold-contaminated seeds are more friable than healthy seeds and, therefore, more susceptible to physical damage (27). Mycotoxin-contaminated dust particles may be inhaled by exposed farmers, barge personnel, and elevator workers. Increased incidences of chronic bronchitis, asthma, and emphysema have been reported among grain handlers (28, 29). Burg et al. (15) detected aflatoxins in corn dust generated on farms, and zearalenone and secalonic acid D have been identified as contaminants in corn dust collected from terminal grain storage elevators in the New Orleans area that handle corn from the midwestern United States (14, 19). Zearalenone and vomitoxin, mycotoxins formed by *Fusarium* species, usually occur together in contaminated grains (30, 31). Palmgren et al. (19) isolated colonies of toxigenic strains of *Aspergilli, Penicillia,* and *Fusaria* in this grain dust. Among these strains were *P. oxalicum, P. viridicatum, A. flavus, F. graminearum,* and *F. moniliforme,* mold species capable of producing secalonic acid D, ochratoxins, aflatoxins, vomitoxin, and zearalenone, respectively.

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METHODS

Apparatus

Equipment specified is not restrictive; other suitable equipment may be substituted.

(a) *Fluorodensitometer.*—Schoeffel SLD (Schoeffel Instrument Div , 24 Booker St, Westwood, NJ 07675). Excitation at 365 nm and emission at 425 nm for aflatoxins and ochratoxin.

(b) *Liquid chromatograph.*—Model ALC 502/401 (Waters Associates, Milford, MA 01757) equipped with 6000A pump and U6K septumless injector. Injector was connected to Whatman (9 Bridewell Pl, Clifton, NJ 07014) guard column (7 cm, id = 2.1 mm) packed with Perisorb RP-18, 30–40 μm (EM Laboratories, Inc., 500 Executive Blvd, Elmsford, NY 10523).

(c) *LC column.*—Reverse phase, Partisil-10 ODS 2 (Whatman), 4.60 mm id × 250 mm long.

(d) *Detectors.*—Waters Model 440 and 441 ultraviolet detectors operated at 340 nm for secalonic acid D, 280 nm for zearalenone, and 214 for vomitoxin.

(e) *Chart recorder.*—Omniscribe dual pen, Model A5213-2 (Houston Instrument, 1 Houston Sq, Houston, TX 78753).

(f) *Wrist-action shaker.*—Burrell, Pittsburgh, PA 15219.

(g) *Filtration bell.*—Fisher Filtrator (low form) (Fisher Scientific Co., Springfield, NJ 07081).

(h) *Thin layer chromatographic (TLC) plates.*—Aflatoxin and ochratoxin—glass plates 20 × 20 cm, coated with 0.5 mm wet layer Adsorbisil-1 silica gel (Applied Science Laboratories, State College, PA 16801). Activate 1 h at 100–110°C. Score before using.

(i) *Plate scorer.*—Schoeffel.

(j) *Viewing cabinet.*—Chromato-vue (Ultra-Violet Products, Inc., San Gabriel, CA 91778), fitted with two 15 watt long wavelength lamps.

(k) *Sample vials.*—2 dram with polyethylene snap caps. Also, 16 × 125 mm culture tubes with Teflon-lined Bakelite caps. Vials and tubes were nitric acid-washed and oven-dried (180°C) before use.

(l) *Chromatographic columns.*—Glass Econocolumn (1 × 30 cm) (Bio-Rad, PO Box 708, Rockville Center, NY 11571).

(m) *Syringes.*—Hamilton 7100 series for spotting TLC plates, 800 series for LC injections. Also, disposable 1 mL plastic syringes with barrel removed, as reservoir for Sep-Pak cartridges.

(n) *Sep-Pak.*—RP-18 silica cartridge (Waters) prepared for use by washing with 2 mL methanol followed by 10 mL water.

(o) *Centrifuge.*—Damon/IEC HN-S II with 24-place angle rotor (International Equipment Co., Needham Heights, MA).

Reagents

(a) *Solvents.*—Reagent grade methanol, methylene chloride, hexane, ethyl acetate, acetonitrile, toluene, acetic acid, and acetone. LC grade acetonitrile and tetrahydrofuran. Water purified by Milli-Q system (Millipore, Bedford, MA 01730).

(b) *Sodium bicarbonate solution.*—4 g sodium bicarbonate in 100 mL water.

(c) *Phosphoric acid solution (1M).*—6.8 mL concentrated H_3PO_4 diluted to 100 mL with water.

(d) *Sulfuric acid solution (2M).*—28 mL concentrated H_2SO_4 diluted to 1 L with water.

(e) *Citric acid solution.*—700 g in 1 L water.

(f) *Sodium hydroxide solution.*—40 g NaOH in 1 L water (1M).

(g) *Activated carbon.*—Darco G60 (J. T. Baker Chemical Co.).

(h) *Neutral alumina.*—Chromatographic grade (No. 9296, MCB).

(i) *Diatomaceous earth.*—Acid-washed Celite 545 (Johns Manville).

(j) *Developing solvents for TLC.*—Aflatoxins: ethyl ether–methanol–water (96 + 3 + 1); ochratoxin A: toluene–acetic acid (95 + 5).

(k) *LC elution solvents.*—Secalonic acid D and zearalenone: acetonitrile–water–tetrahydrofuran–acetic acid (4 + 3 + 0.5 + 0.5), pH 2.8; vomitoxin: acetonitrile–water (1.5 + 8.5).

(l) *Standards.*—Aflatoxins and ochratoxin A were obtained from Makor Chemicals, Ltd, Jerusalem, Israel. Purity of each was established by TLC. Zearalenone was a gift from N. Bachman, Commercial Solvents, Inc., Terre Haute, IN. Vomitoxin was prepared from fermentation of *F. graminearium* NRRL 5883 on corn and isolated by the method of Vesonder et al. (20). Secalonic acid D was purified in our laboratory from YES medium inoculated with *P. oxalicum* according to the method of Ciegler et al. (32). Purity of vomitoxin and secalonic acid D was established by melting points, nuclear magnetic resonance spectra, and elution as single peaks on liquid chromatograms. Standard solutions were prepared in ethanol and concentrations were determined using Beer's law from known values of ϵ (33) (aflatoxin B_1, ϵ(362 nm) = 21 800; aflatoxin B_2, ϵ(362 nm) = 23 400; aflatoxin G_1, ϵ(362 nm) = 16 100; aflatoxin G_2, ϵ(363 nm) = 21 000; ochratoxin A, ϵ(333 nm) = 6400; zearalenone, ϵ(274 nm) = 13 900; secalonic acid D, ϵ(340 nm) = 31 200; vomitoxin, ϵ(217 nm) = 5220), molecular weights, and ultraviolet absorption. Solutions were stored in the dark at −20°C. Solutions were prepared to contain 7.4 μg aflatoxin B_1, 1.4 μg aflatoxin B_2, 3.2 μg aflatoxin G_1, 2.9 μg aflatoxin G_2, 4.2 μg ochratoxin A, 43.8 μg zearalenone, 45.2 μg secalonic acid D, and 205 μg vomitoxin/mL.

(m) *Grain dust.*—Dust was collected from various locations within 2 terminal grain elevator facilities on the Mississippi River in the New Orleans area. Dust was also collected from the dust control system (baghouse) of an elevator in Ohio. The dust was mainly from corn although some samples were from soybean and wheat. For our study a composite dust was prepared containing 4 parts corn dust, 1 part soybean dust, and 1 part wheat dust. Samples were sifted through a 40 mesh screen to remove particles larger than 420 μm. At least 65% of the remaining particles were smaller than 70 μm. The dust was stored at −4°C in air-tight plastic bags held in metal cans. Before extraction the dust was spiked with aliquots from the standard solutions, shaken for 30 min at room temperature, and allowed to stand overnight before extraction.

Extraction

Weigh 50 g dust in 500 mL Erlenmeyer flask. Add 25 mL 1M phosphoric acid and 250 mL methylene chloride. Shake 30 min on wrist-action shaker; filter through fluted paper and press dry with 50 mL round-bottom flask. Collect ≥ 200 mL filtrate. Transfer three 50 mL aliquots into separate 100 mL Erlenmeyer flasks with glass stoppers for aflatoxin, ochratoxin A, and combined zearalenone and secalonic acid assays. (The remaining 50 mL may be reserved to repeat assay, if necessary.) Return filter cake to 500 mL Erlenmeyer flask for vomitoxin extraction.

With one 50 mL aliquot, proceed with aflatoxin assay by Lee and Catalano (34) modification of official AOAC method (10). For ochratoxin assay, transfer 50 mL aliquot of filtrate to 250 mL separatory funnel. Add 70 mL bicarbonate solution and shake 1 min. After phase separation, drain lower methylene chloride layer into second 250 mL separatory funnel. Add additional 35 mL bicarbonate solution and shake. Discard lower (methylene chloride) layer and acidify to pH 3.5 with 2M sulfuric acid (ca 20 mL). Transfer acidified solution to separatory funnel, add 50 mL methylene chloride, and shake carefully, because there will be some additional carbon dioxide evolution. Drain lower methylene chloride layer through sodium sulfate (2.5 cm in Butt tube) into 250 mL beaker. Re-extract aqueous phase with additional 50 mL methylene chloride and drain through sodium sulfate. Evaporate methylene chloride extract to near dryness on steam bath. Transfer quantitatively to glass vial and evaporate solvent under stream of nitrogen. Reserve sample for TLC analysis.

For isolation of zearalenone and secalonic acid D, add 50 mL sodium hydroxide solution to 50 mL aliquot of methylene chloride extract in 250 mL separatory funnel, let layers separate, discard lower methylene chloride layer. Add 50 mL citric acid solution to neutralize base and then re-extract with 50 mL methylene chloride. After layers separate, repeat extraction with additional 50 mL methylene chloride and then, after drying combined methylene chloride extracts with sodium sulfate as above, evaporate to near dryness on steam bath. Transfer residue to vial with ethyl acetate and re-evaporate under stream of nitrogen. Redissolve residue in 200 μL acetonitrile and add 800 μL water. Transfer to RP-18 Sep-Pak cartridge fitted with 1 mL disposable plastic syringe with barrel removed. Centrifuge 5 min at 1000 rpm (collect filtrate in small plastic centrifuge tube) and discard filtrate. Then elute secalonic acid D and zearalenone from cartridge with two 500 μL portions of acetonitrile–water (7 + 3) and again use centrifugation to recover eluate. Save for LC determination of secalonic acid D and zearalenone.

To isolate vomitoxin, extract filter cake, which was saved after initial phosphoric acid–methylene chloride extraction, with 100 mL acetonitrile–water (86 + 14) by shaking for 30 min. Filter through fluted paper; collect ≥ 50 mL. Prepare charcoal–alumina–diatomaceous earth column as described by Trucksess et al. (35), substituting glass Econocolumns for polypropylene columns. Support column, fitted with 3 in. metal cannula passing into 50 mL beaker, in glass bell filtration apparatus connected to vacuum (ca 20 torr). Pass 25 mL acetonitrile–water extract through column. Wash with additional 10 mL acetonitrile–water (86 + 14). Evaporate ethyl acetate to dryness and redissolve extract in 100 μL acetonitrile. Add water to 2 mL volume. Pass through silica cartridge by centrifugation as described above. Discard eluate. Wash cartridge twice with 500 μL acetonitrile–water (3.5 + 6.5) to elute vomitoxin. Save for LC determination.

Quantitation

TLC.—Dissolve evaporated aflatoxin sample extract in 1 mL benzene–acetonitrile (98 + 2). Spot 5 μL aliquots of sample extract on scored TLC plate adjacent to aliquots of standards ranging from 5 to 20 ng per spot. Develop plate in

Table 1. Recovery (%) and lowest detectable levels (μg/kg) of mycotoxins added to grain dust

Mycotoxin	Added, μg/kg	Rec., %	SD, %[a]	Lowest detectable level, μg/kg
Aflatoxin B_1	0.5–40	76	23	<0.5
B_2	0.5–40	83	21	<0.5
G_1	0.5–40	77	24	<0.5
G_2	0.5–40	57	23	<0.5
Ochratoxin A	1–100	88	16	10
Zearalenone	10–1000	81	15	20
Secalonic acid D	5–400	98	29	10
Vomitoxin	5–400	84	29	10

[a]Triplicate samples were analyzed at each of 4 spiking levels.

ether–methanol–water (96 + 3 + 1) ca 30 min. Scan plate in densitometer and calculate aflatoxin content from peak areas of samples and standards (10, 34). Dissolve samples for ochratoxin analysis in 100 μL benzene–acetonitrile (98 + 2) and spot 5 μL samples as well as aliquots of standards ranging from 20 to 400 ng per spot. Develop plate in toluene–acetic acid (95 + 5), air-dry, and then scan plate in densitometer and calculate ochratoxin concentration as for aflatoxins.

LC.—(a) *Secalonic acid D and zearalenone.*—Prepare mobile phase of acetonitrile–water–tetrahydrofuran–glacial acetic acid (4 + 3 + 0.5 + 0.5) and filter through 0.45 μm Teflon filter. Degas filtrate by evacuation at 20 torr for 30 min. Using 2 LC ultraviolet detectors in tandem, set one for absorption at 280 nm and the other for absorption at 340 nm. Stabilize system at 1.7 mL/min (1500 psi). Inject 20 μL aliquots with detector set at 0.01 or 0.05 absorbance unit full scale (AUFS). Measure peak heights for zearalenone (A_{280} detection; k' = 3.39; at 0.01 AUFS, 0.59 ng/mm peak height) and secalonic acid D (A_{340} detection; k' = 4.09; at 0.01 AUFS, 0.62 ng/mm peak height). To verify, inject 20 μL sample plus 5 μL standard (43.8 μg zearalenone or 49.4 μg secalonic acid D/mL). Differences between peak heights of samples plus standard and that of the standard should equal peak heights of samples alone. Calculate concentrations from peak heights of samples compared with standards. Alternatively, use peak areas of samples and standards to compute concentrations of unknown. (This way is more accurate but is only possible if peak separations are sufficient to allow accurate area computation. Usually peaks are sufficiently sharp to allow peak heights to accurately reflect amounts.) (b) *Vomitoxin.*—Prepare mobile phase of acetonitrile–water (1.5 + 8.5). Filter and degas as described above. Set Model 441 detector at 214 nm and stabilize LC system at 1.5 mL/min (vomitoxin elutes at k' = 3.30; at 0.01 AUFS, 1.46 ng/mm peak height). Inject 20 μL aliquots with detector set at 0.01 AUFS. Co-inject sample and standard to verify peak migration and quantitation. Calculate concentration from peak height of sample compared to peak height of standard.

Results and Discussion

The multimycotoxin method described in this report was designed to use separate aliquots of the extract for determination of all mycotoxins except zearalenone and secalonic acid D which were recovered and analyzed simultaneously. The method takes advantage of the use of both TLC and LC to maximize sensitivity of detection and simplicity of approach. Aflatoxins and ochratoxin, which are highly fluorescent under ultraviolet illumination, are detected with great sensitivity on TLC plates (10, 11). The other toxins studied are not detected with great sensitivity by TLC and, therefore, an LC approach was chosen to analyze these toxins. Aflatoxins were determined by standard methods (10) using silica column chromatography for initial cleanup. Ochratoxin was separated from the mixture by extraction into sodium bicarbonate solution, and secalonic acid D and zearalenone by extraction into dilute sodium hydroxide solution. Acetonitrile–water reextraction of the residue was used to recover vomitoxin from methylene-chloride extracted, spiked grain dust. Separate analysis of the methylene chloride extract showed that less than 10% of the total vomitoxin was obtained in this extract. Therefore, most of the added vomitoxin remained in the residue. The extraction of grain dust with methylene chloride aided the cleanup of vomitoxin before LC analysis. Overall recoveries of the mycotoxins from spiked grain dust are given in Table 1. Inclusion of phosphoric acid was necessary for good recovery of the acidic mycotoxins, zearalenone, secalonic acid D, and ochratoxin, but may have slightly reduced the recovery of aflatoxins. Other studies using a similar TLC approach have reported higher recoveries for aflatoxins (5, 8).

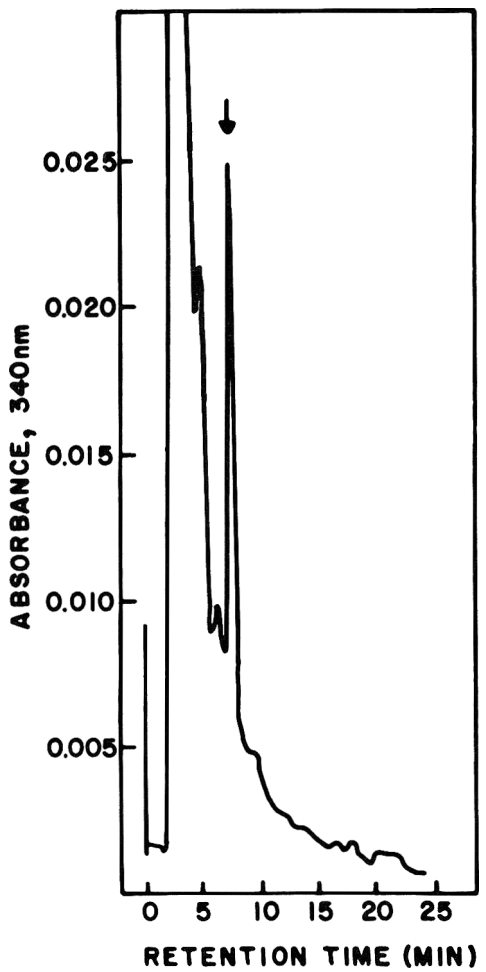

Figure 1. Liquid chromatogram of secalonic acid D (arrow) recovered from grain dust spiked with 200 ppb standard (340 nm).

The dust used for these spiking studies consisted mainly of corn matter but also contained dust from soybean and wheat. All of the particles were less than 420 μm; 65% passed through a 70 μm sieve. The dust had been previously analyzed for zearalenone, aflatoxin, ochratoxin, and secalonic acid D by the Palmgren et al. (19) and Ehrlich et al. (14) single-mycotoxin methods. Only secalonic acid D was present at a concentration of 60 ppb. However, other dusts examined from New Orleans terminal storage and shipping elevator have been shown to contain zearalenone and secalonic acid D at levels as high as 50 ppm (14, 19). The presence of zearalenone

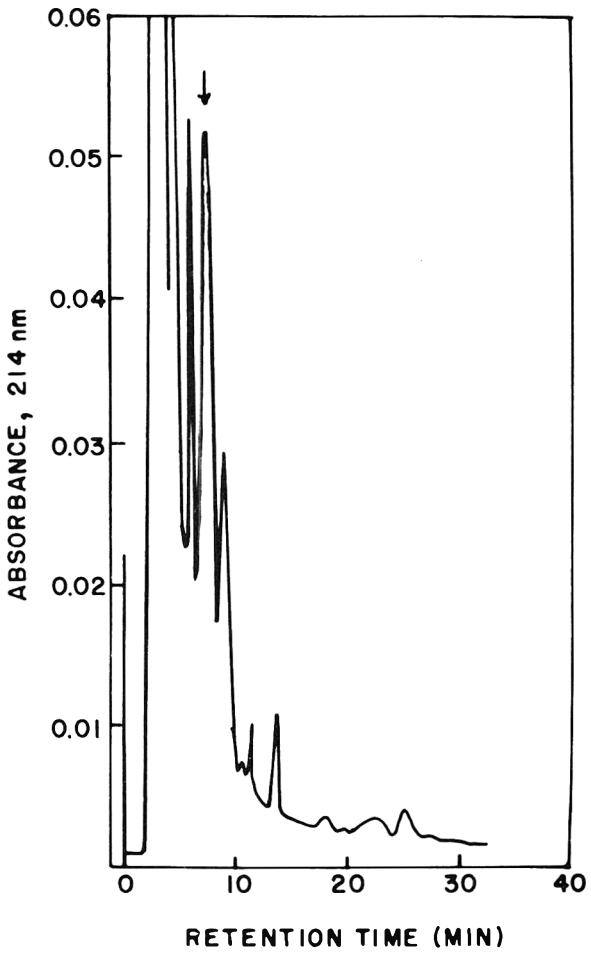

Figure 2. Liquid chromatogram of vomitoxin (arrow) recovered from grain dust spiked with 200 ppb standard (214 nm).

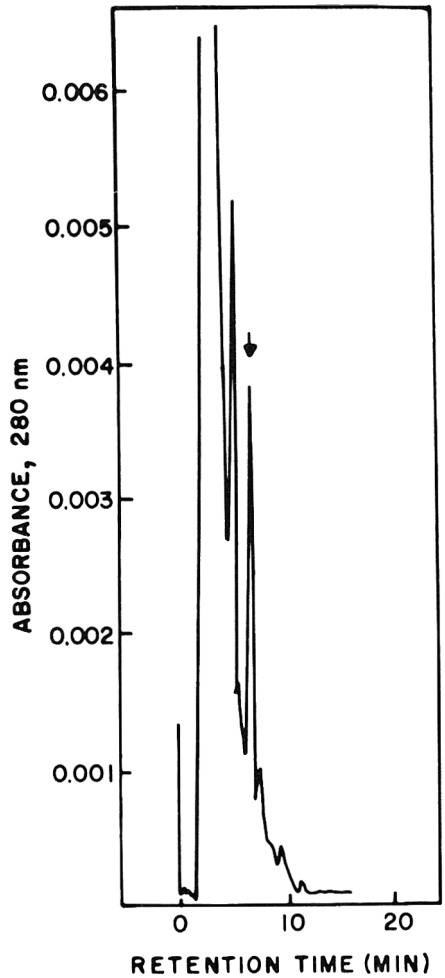

Figure 3. Liquid chromatogram of zearalenone (arrow) recovered from grain dust spiked with 100 ppb standard (280 nm).

in some samples of dust suggested that vomitoxin may also be a co-contaminant because both can be produced by the same mold *(F. graminearum)* (30, 31). Preliminary data show that vomitoxin is indeed a contaminant of some samples of dust; these data will be reported separately. Aflatoxins have been reported as contaminants in grain dust (15, 17) but were not present in any of the samples collected from local elevators that mainly store grains from the midwestern United States. The single-toxin methods used for the previous studies were time-consuming and, especially for zearalenone, were not particularly sensitive because a colorimetric determination on TLC plates had been used. Development of a multimycotoxin method allowing quantitation of all these toxins and retaining sensitivity in the ppb range was, therefore, desired. The method described here has been applied to 13 samples, and required approximately 4 days to prepare, extract, and analyze all samples.

The recovery of aflatoxins and ochratoxin A followed methods previously described (10, 11, 34). These cleanup and TLC approaches gave good separation from other fluorescent compounds in the mixture. However, at the lowest spiking level (10 ppb) for ochratoxin, some smearing of the band occurred, which was corrected for when recoveries were estimated. Using this method, therefore, the lower limit for detection of ochratoxin A is about 10 ppb.

Vomitoxin cleanup before LC analysis involved the Trucksess et al. method (35) as modified in the Romer procedure (36). A further silica cartridge cleanup step was added before LC analysis to eliminate some impurities and to remove insoluble matter in the sample. The sample for vomitoxin analysis was applied to the activated silica cartridge in acetonitrile–water (2.5 + 97.5); the cartridge retained more than 99% of the vomitoxin. Vomitoxin was eluted from that cartridge with acetonitrile–water (3 + 7), conditions which allowed recovery of 93% of the added vomitoxin (demonstrated in control studies using purified vomitoxin).

Cartridge cleanup before LC analysis was also used for secalonic acid D and zearalenone. Initially, both secalonic acid D and zearalenone were partitioned into dilute sodium hydroxide solution and then recovered into methylene chloride after using citric acid to neutralize the base. The extraction and quenching into citric acid were carried out as rapidly as possible to avoid possible hydrolysis of the ester group of secalonic acid D. In control studies, recovery of secalonic acid was not affected by extraction into base when neutralization occurred within 10 min of the extraction. For silica cartridge cleanup, elution of the adsorbed material with acetonitrile–water (7 + 3) allowed >95% recoveries of secalonic acid and zearalenone. Because recovery from the cartridge cleanup step was almost quantitative, most of the reduction in recovery of secalonic acid D and zearalenone (Table 1) occurred in the initial extraction step. LC of zearalenone and secalonic acid was attempted both with and without cartridge cleanup. Cleanup eliminated some slow-eluting impurities, allowed somewhat better resolution of the secalonic acid peak (Figure 1), and eliminated a filtration step usually needed before LC analysis. Using centrifugation to recover the eluate, rather than pressure, assured complete recovery and allowed

use of less solvent for the elution. In the procedures described above we found it possible to reuse the silica cartridges, if, after obtaining the sample, each cartridge was washed with 5–10 mL methanol and then with 10–20 mL water.

LC analyses of vomitoxin (Figure 2), zearalenone (Figure 3), and secalonic acid D (Figure 1) were on the same reverse phase silica column, thereby eliminating the column preparation time required for analysis if different LC columns were used. With secalonic acid D and zearalenone, the same LC solvent system was used, thus allowing simultaneous analysis of these 2 toxins. Under these conditions, with tandem monitoring at 340 and 280 nm, both secalonic acid D and zearalenone can be quantitated in a single run. At 340 nm, zearalenone had 11% of the absorbance per ng as secalonic acid, while at 280 nm, secalonic acid had 40% of the absorbance of zearalenone.

The methods given here for LC analysis do not allow complete resolution of the mycotoxin peaks from other impurities (Figures 1–3). However, the peaks are sufficiently sharp to allow quantitation from peak height measurements. With zearalenone and vomitoxin, grain dust extracts prepared in the absence of added toxin did not give peaks eluting at the positions shown. Recoveries (Table 1) compared favorably with those reported for other methods, although reproducibility was somewhat lower. For vomitoxin, the limit of detection is almost equal to those found for most other methods previously described using gas chromatography (37). The percent recoveries were similar for all toxins except secalonic acid D. Secalonic acid D was detected in control, unspiked samples of the same dust at a concentration of 60 ppb. This background amount has been subtracted to calculate results given in Table 1. The high average recovery of secalonic acid may, in part, reflect an underestimation of the amount of secalonic acid in the original sample. Recoveries of toxin were similar over the entire range of spiking amounts tested. The levels chosen for spiking reflect the amounts of the individual toxins reported as contaminants in corn (14, 16–18, 20).

It is usual practice to screen samples for single toxins; in the past, the mutagenic toxins, aflatoxins B_1 and G_1, have received the major emphasis. The method presented here allows single-toxin screening as well as multitoxin screening because individual cleanup steps are used for the different toxins. More and more studies are reporting that mold-contaminated foods and feeds contain more than one toxin and that these multiple toxins can exert their biological effects in a synergistic manner (2, 38, 39). Therefore, it is increasingly important to subject samples to screening procedures for suspected multiple toxic contaminants.

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Rapid, Sensitive Liquid Chromatographic Method for Determination of Zearalenone and α- and β-Zearalenol in Wheat

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A rapid, sensitive liquid chromatographic (LC) method is described for quantitative determination of zearalenone and α- and β-zearalenol in wheat. The procedure incorporates an internal standard, zearalenone oxime, to facilitate quantitation and automated analysis. A sample, buffered with pH 7.8 phosphate, is extracted with water–ethanol–chloroform (2 + 50 + 75) and cleaned up. The final residue is dissolved in LC mobile phase and injected onto a reverse phase RP-18 column under the following conditions: water–methanol–acetonitrile (5 + 3 + 2) mobile phase; fluorescence (excitation wavelength 236 nm, 418 nm cut-off emission filter) and UV (254 nm, range 0.0025 AU) detectors. The limit of detectability (twice background) is 0.5 ng for zearalenone and α-zearalenol standards on the fluorescence detector and 4 ng for β-zearalenol on the UV detector, which is equivalent to 20 μg zearalenone and 20 μg α-zearalenol/kg, and 160 μg β-zearalenol/kg feed. Standard curves are linear over the range 0–35 ng zearalenone and α-zearalenol on the fluorescence detector and 0–50 ng β-zearalenol on the UV detector. Recoveries of all compounds are 87.5–101% in the range 0.1–3.0 mg/kg (ppm).

During the winter wheat harvest in July 1980, Ontario farmers observed a great deal of sprouting and pink discoloration of the wheat kernels (). As a direct result of germination and mold growth, wheat delivered to the elevators was downgraded. Subsequently, the mold contamination on wheat samples was identified as *Fusarium graminearum* (2). *Fusarium* molds, indigenous to the temperate Canadian climate, produce a variety of mycotoxins, depending on growth conditions (3–7).

The presence of mold contamination on the wheat crop created an urgent need to develop rapid and sensitive methods for analyses of these potentially harmful metabolites. A current major concern is the identification and quantitation of the mycotoxin zearalenone (and related metabolites), present in trace amounts in feed samples (8, 9).

Zearalenone [(E.S)-2,4-dihydroxy-6-(6′-oxo-10′-hydroxy-1-undecenyl)benzoic acid-μ-lactone], α-zearalenol [(E,R,S)-2,4,dihydroxy-6-(6′,10′-dihydroxy-1-undecenyl)benzoic acid-μ-lactone], and β-zearalenol [(E,S,S)-2,4-dihydroxy-6-(6′-10′-dihydroxy-1-undecenyl)benzoic acid-μ-lactone] (Figure 1) are *Fusarium* metabolites. All 3 may cause reproductive problems in swine (10–14). Zearalenone acts systematically as an estrogenic substance (15–18). The feeding of *Fusarium*-contaminated diet to swine resulted in prolonged estrus in females, a lack of libido in boars, and no conceptions during a 4-month feeding trial (19). In swine, zearalenone has been associated with abortion, mummified fetuses, stillbirths, reduced litter size, "splaylegs," incoordination of the hind limbs, and fewer viable animals at birth (20–25).

Numerous methods are available for determination of zearalenone in plant material (26, 27), e.g., thin layer chromatography (TLC) (28–31), minicolumn chromatography (32), gas chromatography (GC) (33–39), mass spectrometry (MS) (31, 33–36), liquid chromatography (LC) (40–49), and bioassays (19, 50). To assess the safety of contaminated plant products, it is necessary to analyze zearalenone, as well as α- and β-zearalenol, at the parts per billion (ppb) level. This paper describes a sensitive LC method for quantitative analysis of zearalenone and α- and β-zearalenol in wheat, which incorporates an internal standard, zearalenone oxime [(E,S)-2,4-dihydroxy-6-(6′-oxo-10′-hydroxy-1-undecenyl)benzoic acid-μ-lactate oxime], to facilitate quantitation and automated analysis.

METHOD

Apparatus

(a) *Glassware.*—Before use, thoroughly wash pipets and volumetric flasks with detergent, tap water, distilled water, and methanol. Wash other glassware before use with detergent, tap water, and distilled water, followed by heating in muffle furnace ≥ 2 h at 500°C.

(b) *Vortex mixer.*—Vortex-Genie (Fisher Scientific Co., Ottawa, Ontario).

(c) *Clinical centrifuge.*—Dynac II centrifuge (Fisher Scientific Co.).

(d) *Liquid chromatograph.*—Spectra-Physics Model SP-8000-03, microprocessor-based. Liquid chromatographic system fitted with temperature-programmed oven (set at 30°C); auto-injector with high pressure injection valve and 250 μL loop, Model A0280-020; ternary gradient system with degas, Model A0802-010; single channel data system, Model A0475-010; 16 K RAM memory, Model A0655-010; detector autozeroing device, Model A1644-010, to upgrade system to SP-8000B; and cathode ray tube display unit, Model A1645-010. Dual channel printer/plotter, Model A0275-020; second data system channel, Model A0555-010; syringe injector kit, Model A0346-010; and autosampler, Model SP8010 (Technical Marketing Associates Ltd, Ottawa, Ontario).

(e) *LC column.*—Stainless steel (25 cm × 4.6 mm id), packed with reverse phase RP-18, 5 μm OD-5A Spheri-5 (Brownlee Labs, Santa Clara, CA). Guard column, Part No. 84550 (Waters Scientific Ltd. Mississauga, Ontario), filled with Spherisorb S5 ODS, 5 μm OD 5, Cat. No. 1610 0310 (Phase Separations, Hauppage, NY), is inserted between injector and LC column.

(f) *Ultraviolet (UV) detector.*—Spectra-Physics Model A0260-010. SP-8210 UV detector with 254 nm filter; range 0.0025 AU.

(g) *Fluorescence detector.*—Schoeffel Model FS-970 liquid chromatography fluorometer with excitation wavelength set at 236 nm, and 418 nm cut-off emission filter (Schoeffel Instrument Corp., Westwood, NJ 07675). Range: 0.01 μA; time constant set at maximum.

(h) *Use of autosampler for LC injection.*—Fill 12 × 75 mm disposable borosilicate glass culture tubes with 4–5 mL analysis sample dissolved in mobile phase solvent (water–methanol–acetonitrile, 5 + 3 + 2). Place tubes in racks provided, alternating sample and flush tubes. Fit polyethylene caps on each tube. Use 250 μL injection loop (dead volume of sipper needle to loop was 44 μL). Prime pump system with above solvent, ensuring all air bubbles are removed. Manually advance the sipper needle to first sample vial. Input parameter set conditions as follows:

Mobile phase, water–methanol–acetonitrile (5 + 3 + 2) premixed
Initial delay, 1 min
Injection delay, 4.2 min

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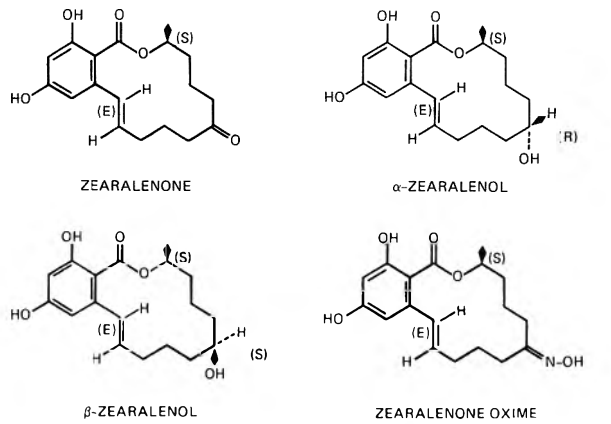

Figure 1. Chemical structures for zearalenone, α-zearalenol, β-zearalenol, and zearalenone oxime.

Run time, 22 min
Number of cycles, input number of samples to be run
Flow rate, 1.8 mL/min
Flow mode, QI or constant flow, isocratic
Oven temperature, 30°C
Report file, input information identifying sample, operator, detectors used, column type, mobile phase
Graph file, identify channel used to acquire and reduce data; Channel 1: UV detector, attenuation × 2, chart speed 1.0 cm/min, zero baseline offset 15%, plotted on right side, intensity 4; Channel 2: fluorescence detector, attenuation × 6, chart speed 1.0 cm/min, zero baseline offset 15%, plotted on left side, intensity 4
Data systems, input data system files to be used with detector channels 1 or 2
Fill time, 4.0 min
Flush time, 10 min
Repetitions per vial, 2

Begin parameter set No. 1 execution—Input "SB1." Enter D(1) Z1 and D(2) Z1. Baseline detector level is taken as zero for the chromatogram. This occurs for each injection, resetting zero points at the same pen position for each chromatogram automatically.

(i) *Shaker.*—Magni Whirl® constant temperature bath (Blue M Electric Co., Blue Island, IL) set at room temperature and high-speed shaking.

(j) *Flash-evaporator.*—Rotating, vacuum, all-glass, 2 L capacity (Buchler Instruments, Fort Lee, NJ).

Reagents

(a) *Solvents.*—2-propanol, 95% ethanol, methanol, benzene, acetonitrile, chloroform, all LC or ACS reagent grade and redistilled in glass in our laboratory. Ether redistilled before use. Glass-distilled chloroform stabilized by addition of 0.75% ethanol.

(b) *Base-acid extraction solvents.*—0.184N NaOH prepared by diluting certified standard 1.002N NaOH (carbonate-free) (Fisher Scientific Co.); 0.50N acetic acid in double-distilled water prepared by diluting certified ACS grade (99.7%) glacial acetic acid (Fisher Scientific Co.).

(c) *LC mobile phase.*—Water–methanol–acetonitrile (5 + 3 + 2). Methanol and acetonitrile (LC grade) were filtered through Millipore filter FH-0.5 μm; water was double-distilled in glass and filtered through Millipore filter HA-0.45 μm (Millipore Corp., Bedford, MA 01730).

(d) *Zearalenone, zearalenone oxime, and α- and β-zearalenol standard solutions.*—Obtained from International Minerals and Chemical Corp., Terre Haute, IN. The α- and

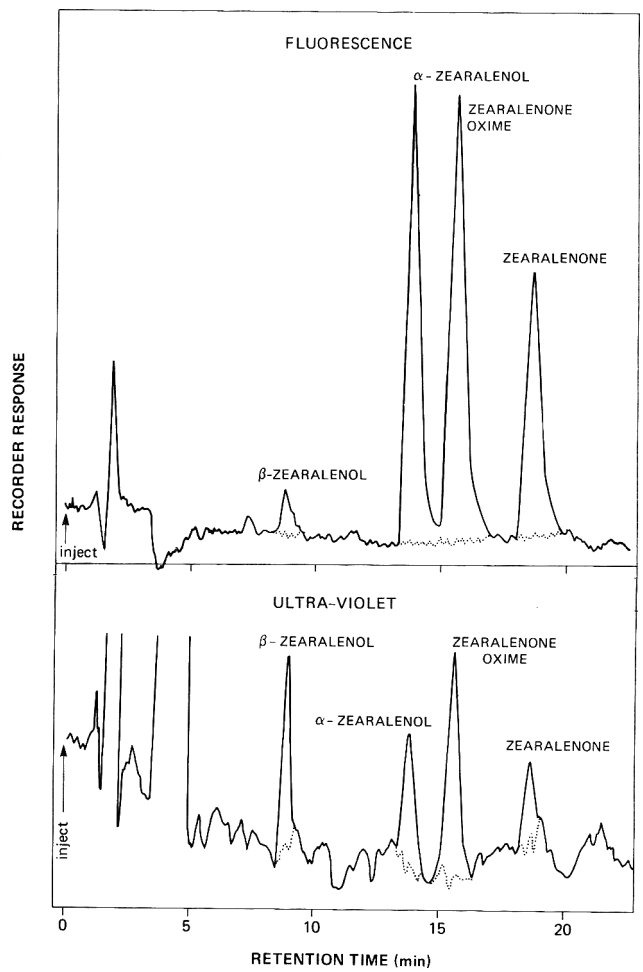

Figure 2. Fluorescence and UV/visible chromatograms of zearalenone (15 ng), α- and β-zearalenol (15 ng), and zearalenone oxime (30.6 ng) standards.

β-zearalenol were also synthesized by sodium borohydride reduction of zearalenone, according to published methods (general ketone reduction reaction) (51). All standards were purified by using LC preparative chromatography. Partisil M9 10/25 OD S-2, C-18, high-loading preparative column (Whatman, Inc., Clifton, NJ) was used to purify standards (mobile phase water–methanol–acetonitrile (9 + 7 + 4); attenuation × 160; balance 0.08 on UV detector; flow rate 6.0 mL/min). Purified standard fractions were evaporated over nitrogen to remove acetonitrile and methanol, then chilled overnight at 4°C and centrifuged at 4°C. The supernate was removed and the residue was dried under vacuum 2 h at 65°C.

Standards were developed on TLC plates impregnated with 10% silver nitrate; mobile phase was 15% methanol in chloroform. All standards gave single spots when viewed under UV light or sprayed with 10% phosphomolybdic acid, except β-zearalenol gave 3 spots. β-Zearalenol was further purified on 250 mm Redicoat AG silica gel G with 10% silver nitrate and then on cellulose plates to remove finely divided silver, which passed through the sintered glass crucible. Sylated derivatives of all standards with MSTFA yielded single peaks on a 3.7 m × 0.64 cm od (2 mm id) glass gas chromatographic column containing 3% OV-17 on 120–140 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, PA) at 258°C, flow rate 25 mL/min (Perkin-Elmer Model 900, Montreal, Quebec). Retention times were α-zearalenol, 11.05 min; β-zearalenol, 11.74 min; zearalenone, 14.45 min; and zearalenone oxime, 15.27 min (39). Positive identification of each

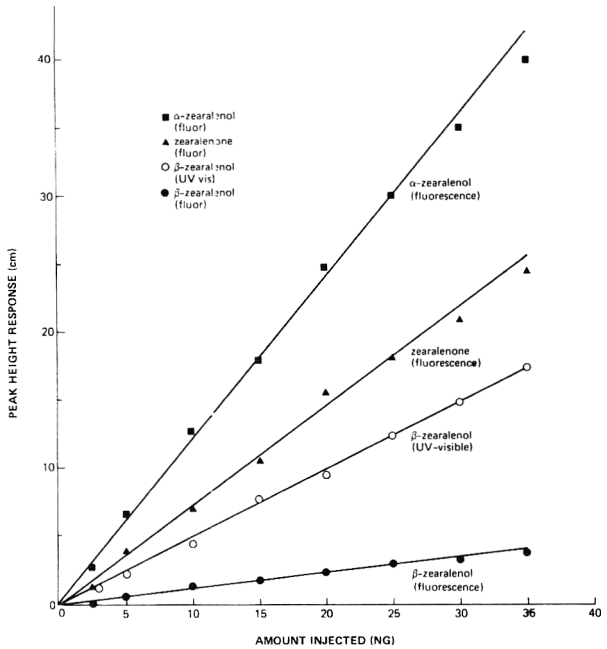

Figure 3. Plot of fluorescence and UV/visible detector responses vs amount of standards injected onto LC column (recorder attenuations: fluorescence, 40 mV; UV/visible, 5 mV).

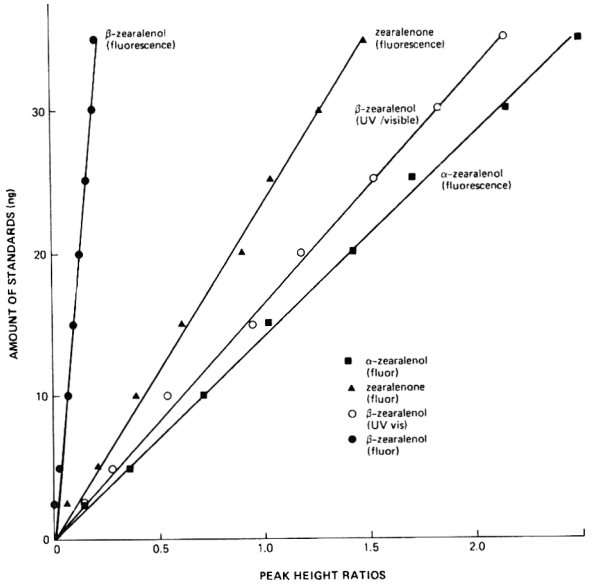

Figure 4. Plot of amount (ng) of standards injected vs ratio of standards/zearalenone oxime peak height ratios (30.6 ng zearalenone oxime added to sample as internal standard). Fluorescence detector (recorder attenuation: 40 mV) used for zearalenone and α- and β-zearalenol; UV/visible detector responses (recorder attenuation: 5 mV) used for β-zearalenol.

mycotoxin was confirmed by mass spectrometry (MS) (Finnigan 3100D, Technical Marketing Assoc., Ltd, Ottawa, Ontario).

Standard solutions were prepared by accurately weighing ca 1 mg each standard and dissolving in 50 mL methanol (20 μg/mL). Two mL aliquots were evaporated to dryness over nitrogen, stored at −30°C, and reconstituted when required. (Peak heights of standards dissolved in methanol decreased 30% over a 10-month period when standards were stored in the refrigerator at 4°C and protected from light.)

(e) *Phenol red indicator.*—10 mg/50 mL water. Color change from red to yellow at pH 8.2–6.0.

(f) *Phosphate buffer.*—Add 8.5 mL 0.2M monobasic sodium phosphate (NaH_2PO_4) to 91.5 mL 0.2M dibasic sodium phosphate (Na_2HPO_4), pH 7.8 (Fisher Scientific Co.).

Analysis of Standards

To establish linearity of LC fluorescence and UV detector responses to zearalenone and α- and β-zearalenol, transfer series of aliquots (50–175 μL) to 25–100 mL volumetric flasks, and dilute each aliquot with water–methanol–acetonitrile (5 + 3 + 2) to prepare standard curves in the range 0–35 ng, the amounts injected from 250 μL loop. Keep amount of internal standard constant at 30.6 ng with each injection. Measure peak heights (fluorescence and UV responses, Figure 2) and plot peak heights vs amounts (ng) of standards injected (Figure 3). Prepare graph (Figure 4) to show the relationship between standards injected (ng) and ratio of peak height responses for respective standard/zearalenone oxime (internal standard) on the same detector.

Cleanup of White Winter Wheat

Grind 600 g representative wheat sample to pass 2 mm sieve and mix thoroughly. Weigh 2.5 g into 500 mL Erlenmeyer flask. Dropwise add 0.2 mL internal standard (12.85 μg zearalenone oxime/mL chloroform) and mix. Add 4.0 mL phosphate buffer (pH 7.8) and shake until buffer is absorbed into wheat sample, i.e., no dry areas remain evident. Use spatula to break up any lumps or caked wheat adhering to sides or bottom of Erlenmeyer flask.

Add 200 mL water–ethanol–chloroform (2 + 50 + 75) and shake vigorously 1 h. Using glass funnels, filter mixture first through clean glass wool plug and then fine 18.5 cm Whatman filter paper No. 5 into 500 mL round-bottom flask. Wash Erlenmeyer flask, residue, and filter paper with two 30 mL portions of extracting solvent and combine extracts. Add 2.0 mL phosphate buffer (pH 7.8), mix, and evaporate to 20–30 mL on rotary evaporator at 40°C. With disposable pipet, transfer concentrate to 100 mL round-bottom flask. Rinse 500 mL flask with 5 mL portions of methanol and add rinses to concentrate. Evaporate on rotary evaporator until volume remaining is ≤ 2 mL. Transfer to 15 mL graduated conical centrifuge tube fitted with Teflon-lined screw cap. Extract remaining residue in 100 mL round-bottom flask with two 4 mL and one 2 mL aliquots of 10% 2-propanol in ether and combine extract in 15 mL centrifuge tube. Mix 2 min on vortex mixer and centrifuge 2 min at 425 × *g*. Transfer upper ether extract to 15 mL round-bottom centrifuge tube fitted with Teflon-lined screw cap. Be careful not to transfer any portion of solid interface.

Place extract on ice 10–15 min and add 2.0 mL chilled 0.184N NaOH (stored in refrigerator at 4°C). Vortex-mix 1 min and then chill on ice. Repeat mixing and chilling. Centrifuge 2 min at 425 × *g* and discard upper layer. Wash cold aqueous layer with 2 mL portions of benzene; avoid formation of an emulsion by very gently inverting tube. Chill and centrifuge. Discard benzene washings.

Add 3 drops of phenol red indicator. Neutralize basic extract to pH 7.0–8.4 range by adding ca 0.85 mL chilled 0.5N acetic acid (4°C) while vortex-mixing. Indicator will change from red to slightly pink. Note: Do not take color change beyond this point to white. Chill and extract 3 times with 2.0 mL benzene each time. Vortex-mix 1 min, chill, and centrifuge 2 min at 1100 × *g*. If emulsion forms, slight lowering of pH will normally break emulsion. Maintain pH at 7.0–8.4.

Evaporate pooled benzene extracts to dryness over stream of nitrogen at 40°C. Add 3.0 mL water–methanol–acetonitrile (5 + 3 + 2) and mix vigorously for 4–5 min on vortex mixer. Dilute 1.0 mL aliquot to 7.0 mL. Inject 0.25 mL onto LC column. Some samples may be slightly cloudy. Use guard

column before LC column, or filter sample through Millipore stainless steel filter device (Cat. No. XX30 012 00, Millipore Corp.) fitted with 0.5 μm organic filter (Cat. No. FHLP 01300) with AP-type prefilter installed on top and held in place with Teflon "O" ring (Millipore Corp.) attached to 5 mL B-D Yale Luerlok syringe (Becton, Dickinson and Co., Rutherford, NJ). Place in freezer at −30°C until day of analysis.

Recoveries of Zearalenone, α- and β-Zearalenol, and Zearalenone Oxime from Wheat

Establish recoveries of α- and β-zearalenol, zearalenone, and zearalenone oxime from 2.5 g-triplicate wheat samples in 500 mL Erlenmeyer flasks at 0.1, 0.5, and 3.0 ppm levels (Table 1). Dropwise add 250, 1250, and 7500 ng standards in 0.20 mL chloroform to separate 2.5 g aliquots of wheat. Add 4.0 mL buffer (pH 7.8) and proceed with cleanup procedure described above. Measure peak heights obtained with those of comparable amounts of standards. Inject amounts in 0–35 ng range to ensure linearity.

Determination

Place sample tubes in SP-8010 autosampler and complete analysis, using parameter set control. Measure peak heights for zearalenone, α- and β-zearalenol, and zearalenone oxime as identified by comparison of retention times with those of standards. Subtract background noise from peak heights. Determine results from standard curves. Multiply results from graph (ng standards) by 3.36×10^{-2} to calculate mg/kg (ppm) levels of zearalenone and α- and β-zearalenol in wheat samples.

Results and Discussion

As more becomes known about nutritional and toxicological effects of zearalenone, greater demands will be placed on the analyst to quantitate trace levels of zearalenone in grains. Recent reports indicate that α- and β-zearalenol may also be present as natural contaminants. Because all derivatives may produce estrogenism, one can expect synergistic effects to occur when animals ingest compounds together. Reproductive problems would occur at much lower levels of zearalenone if α- and β-zearalenol were present in substantial quantities. In such a situation, analysis for zearalenone alone would result in a misleading interpretation.

Liquid chromatography is well suited for zearalenone analysis. With a fluorescence detector, the detection limit for zearalenone and α-zearalenol is 0.5 ng injected. For β-zearalenol, the limit is much higher (4 ng). In our laboratory, we chose to use the UV/visible detector for β-zearalenol (detection limit 4 ng) because a small interfering peak occurred under the β-zearalenol peak when fluorescence detection was used. Our experience suggested that UV/visible detection is more sensitive than the fluorescence detector to solvent flow rates and turbulence in the flow cell. Fluorescence detection of zearalenone/zearalenol eliminates many interfering peaks encountered with UV detection of wheat samples.

Extraction conditions were designed to take full advantage of the solubility characteristics of zearalenone derivatives to minimize interferences, yet maintain high recoveries. LC conditions and sample volumes were selected to achieve good peak resolution and automated analysis.

The internal standard concentration was chosen for analysis of samples in the 0.2–3.0 ppm range. The internal standard eliminates errors due to variability in sample cleanup, injection, and detector response. With no requirement for external standard injections, the time and cost of each analysis are greatly reduced.

Table 1. Recoveries of zearalenone, zearalenone oxime, α-zearalenol, and β-zearalenol from wheat

Added, mg/kg (ppm)	Recovery, %	SD[a]
	β-Zearalenol	
0.1	—	—
0.5	92.8	6.2
3.0	87.5	2.6
	α-Zearalenol	
0.1	90.5	9.4
0.5	91.1	7.9
3.0	91.9	1.4
	Zearalenone Oxime	
0.1	101.0	2.6
0.5	91.4	7.2
3.0	88.1	2.7
	Zearalenone	
0.1	93.5	6.5
0.5	87.9	8.4
3.0	88.6	2.1

[a]All analyses were done in triplicate.

The importance of using contaminant-free solvents and glassware cannot be overemphasized. Using fluorescence and UV/visible LC detectors for analyzing trace mycotoxin levels means that elimination of all interfering peaks is a major objective in method development. Our experience confirmed the advantages of muffling glassware, using solvents redistilled in glass, and washing aqueous solvents with distilled organic solvents to minimize sources of contamination. Samples were generally protected from light sources to avoid decomposition.

A 2.5 g sample of ground wheat was selected; Table 1 reports recoveries. Fifty gram samples were tested, but required large amounts of internal standard. Poorer recoveries (70%) were experienced with the larger samples, probably due to less efficient extractions.

Phase partitioning into 0.1N NaOH and then back into the organic phase by adjusting pH with 0.5N acetic acid was an effective means of removing most interfering peaks present in the wheat, while maintaining high recoveries, provided it is done at low temperatures (4°C). At room temperature, recoveries are generally lower and more variable. The phosphate buffer (pH 7.8) was used to prevent acid-catalyzed degradation of the zearalenone derivatives, especially the oxime. Freshly distilled ether, stabilized with 10% 2-propanol, is an effective solvent for extractions of zearalenone, zearalenone oxime, and α- and β-zearalenol from wheat extracted with 40% ethanol in chloroform. The ether extract does not emulsify when vortex-mixed with aqueous sodium hydroxide. Washing the NaOH extract with benzene must be done very gently to avoid emulsion formation. Because benzene is used in the final extraction, it is advisable to use Teflon-lined screw caps to avoid unnecessary exposure.

Fluorescence and UV detector responses were monitored simultaneously to quantitate all 4 derivatives. By calculating peak ratios for a given zearalenone derivative, one can check the presence of any questionable interferences or artifacts.

Figure 2 shows a typical chromatogram of the standards. The peaks are well resolved and the baseline is stable. Peak pairs (fluorescence and UV) are readily identified for the standards. One exception is β-zearalenol, which possesses poor fluorescence properties. The retention times for the derivatives were β-zearalenol, 8.5 min; α-zearalenol, 13.7

min; zearalenone oxime, 15.4 min; and zearalenone, 18.5 min. Few interfering peaks were observed in the wheat samples.

Standard curves for fluorescence detection were linear in the range of 0–35 ng zearalenone and α- and β-zearalenol. With the UV/visible detector, β-zearalenol curves were linear up to 50 ng. Results (Table 1) suggest that in the range investigated (0.1–3.0 ppm), recoveries of standards and internal standard were comparable and in the range of 87.5–101.0%.

It is difficult for a method to achieve quantitation at such trace levels. To analyze for 3 zearalenone derivatives plus an internal standard, a method must completely resolve all 4 peaks. The cleanup procedure must eliminate contaminants which cause interference for not just one peak, but 4 peaks. Working at trace levels, the analyst is always concerned with injection precision and changes in detector responses which occur during an analytical run. An internal standard procedure is preferred to minimize the latter 2 problems. Finally, a preferred method lends itself to automated LC analysis. Our procedure achieves the objective of quantitating zearalenone derivatives in wheat at trace levels.

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Rapid Quantitation and Confirmation of Aflatoxins in Corn and Peanut Butter, Using a Disposable Silica Gel Column, Thin Layer Chromatography, and Gas Chromatography/Mass Spectrometry

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A simple, rapid, and solvent-efficient method for determining aflatoxins in corn and peanut butter is described. Aflatoxins B_1, B_2, G_1, and G_2 were extracted from 50 g sample with 200 mL methanol–water (85 + 15). A portion of the extract was diluted with 10% NaCl solution to a final concentration of 50% methanol, and then defatted with hexane. The aflatoxins were partitioned into chloroform. The chloroform solution was evaporated, and the residue was placed on a 0.5 g disposable silica gel column. The column was washed with 3 mL each of hexane, ethyl ether, and methylene chloride. Aflatoxins were eluted with 6 mL chloroform–acetone (9 + 1). The solvent was removed by evaporation on a steam bath, and the aflatoxins were determined using thin layer chromatography (TLC) with silica gel plates and a chloroform–acetone (9 + 1) developing solvent. Overall average recovery of aflatoxin B_1 from corn was 82%, and the limit of determination was 2 ng/g. For mass spectrometric (MS) confirmation, aflatoxin B_1 in the extract from 3 g sample (20 ng/g) was purified by TLC and applied by direct on-column injection at 40°C into a 6 m fused silica capillary gas chromatographic column. The column was connected directly to the ion source. After injection, the temperature was rapidly raised to 250°C, and the purified extract was analyzed by negative ion chemical ionization MS.

The CB or AOAC official first action method (1, secs **26.026–26.031**) for the determination of aflatoxins in grains is time consuming and requires the use of several hundred mL solvent/sample. Elements of the CB and BF (1, secs **26.032–26.036**) methods were modified and combined, resulting in a method with improved detection and quantitation and with solvent consumption reduced from 450 to 20 mL/sample.

In the BF method, methanol–water (55 + 45) is used for extraction; however, with this ratio the filtering rate is slow. The filtering rate was improved by increasing the methanol concentration to 85%. The filtrate was diluted with NaCl solution before defatting to decrease the partition of methanol to the hexane phase. Aflatoxins were then partitioned into chloroform and evaporated to dryness. Corn and peanut butter samples with contamination levels greater than 20 ng/g and commodities such as raw peanuts can be analyzed directly by thin layer chromatography (TLC). Other samples require a 0.5 g silica gel column cleanup before TLC analysis.

Aflatoxin B_1 (B_1) identity was confirmed using on-column gas chromatography (GC)/negative ion chemical ionization (NICI) mass spectrometry (MS). The confirmation of B_1 identity by MS has become the preferred method. The techniques used include low resolution electron ionization (2), high resolution multiple ion monitoring with solid probe sample introduction (3), on-column GC/MS with high resolution 2-ion monitoring (4), and MS/MS using solid probe sample introduction (5). The MS/MS technique requires advanced instrumentation that generally has not been available. For other MS techniques, B_1 must be isolated and purified. To this end, preparatory 2-dimensional TLC and column chromatography have been used successfully (6). However, these techniques are time consuming. Recently it has been shown that B_1 standard can be chromatographed by capillary GC using on-column injection (7). The on-column injection technique has been described in detail (8). When this technique was applied, B_1 isolated from a sample extract prepared by this method using one-dimensional TLC separation was clean enough for MS analysis. This results in shorter cleanup time and improved recovery of B_1.

METHOD

Apparatus

(a) *Wrist-action shaker.*—Model BT (Burrell Corp., Pittsburgh, PA 15219).

(b) *Chromatographic tube.*—Polypropylene column, 12 mm id packed with 20 μm frit (No. 7121-6, J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

(c) *Vacuum apparatus.*—Baker extraction system (J. T. Baker Chemical Co.).

(d) *Vials.*—2 dram, with foil-lined screw caps.

(e) *TLC plate.*—20 × 20 cm (E. Merck 5763) silica gel 60, precoated.

(f) *Viewing cabinet.*—Chromato-Vue Model C-6 (Ultra-Violet Products, Inc., San Gabriel, CA 91778), fitted with 15 W longwave ultraviolet (UV) lamp.

(g) *Fluorodensitometer.*—Schoeffel SD 3000 (Schoeffel Instrument Corp., Westwood, NJ 07675).

(h) *Integrator.*—SP4100 (Spectra Physics Inc., Piscataway, NJ 08854).

(i) *Automatic elution system.*—Eluchrom (Camag Applied Analytical Industries, Wilmington, NC 28405).

(j) *Mass spectrometer.*—Finnigan 3300 CI with 9500 GC (Finnigan MAT Corp., San Jose, CA 95134). Operating conditions: scan range 55–655 um, scan time 1.5 s, source pressure 0.7 torr, source temperature 110–130°C, reagent gas methane, filament current 0.5 mA, electron energy 140 eV. Introduce sample onto column with syringe or use commercial on-column injector.

(k) *Concentrator tube.*—Chromaflex (Kontes K-422560, Kontes Glass Co., Vineland, NJ 08360).

(l) *GC column.*—6 m × 0.2 mm id methyl silicone, fused silica.

Reagents

(a) *Solvents.*—Methanol, hexane, chloroform, anhydrous ethyl ether, dichloromethane, acetone, and isopropanol, ACS grade.

(b) *Silica gel.*—40 μm (No. 7024-5, J. T. Baker Chemical Co.).

(c) *Aflatoxins standard.*—Prepare in benzene–acetonitrile (98 + 2) as in secs **26.006–26.011** (1) to contain 0.5 μg B_1 and G_1/mL and 0.15 μg B_2 and G_2/mL for TLC analysis. Prepare in acetone to contain 20 ng B_1/μL for MS confirmation. Store diluted standards in refrigerator in vapor-proof container.

Extraction and Partition

Weigh 50 g ground corn or peanut butter into 500 mL glass-stopper Erlenmeyer flask. Add 200 mL methanol–water (85 + 15) and secure stopper with masking tape. Shake 30 min on wrist-action shaker and filter through medium paper. Collect 40 mL filtrate in 50 mL graduated cylinder. Transfer to

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125 mL separatory funnel. Add 40 mL 10% NaCl solution, mix, and add 25 mL hexane. Shake 1 min. Let phases separate, and drain lower phase into second 125 mL separatory funnel. Discard upper phase. Extract aflatoxins from aqueous phase with two 25 mL portions of $CHCl_3$; shake 1 min each time. Combine $CHCl_3$ fractions in 125 mL Erlenmeyer flask and evaporate extract to dryness on steam bath.

Silica Gel Column Chromatography

Add 0.5 silica gel to chromatographic tube. Place 20 μm frit on top of packing (alternatively, 6 mL disposable extraction column packed with silica gel (Baker 7086-6) can be used). (Note: Silica gel or prepacked columns were used as received from the manufacturer. Variable water content arising from exposure to different humidity conditions was not a problem.) Attach column to Baker extraction system. Condition column by washing with 3 mL hexane, then 3 mL dichloromethane using vacuum (flow rate 6 mL/min). Dissolve sample residue in 3 mL dichloromethane and add to column. Let sample drip freely (flow rate 3 mL/min, apply vacuum if needed). Rinse flask with two 1 mL portions of dichloromethane and add rinse to column. Wash column with 3 mL hexane, 3 mL anhydrous ethyl ether, and then 3 mL dichloromethane (use vacuum, flow rate 6 mL/min). Turn off vacuum, remove extraction system cover, and place 2 dram vial under each column. (Test tube rack can be used to hold vials.) Elute aflatoxins without vacuum with two 3 mL portions of $CHCl_3$–acetone (9 + 1). Evaporate eluate to dryness on steam bath under stream of nitrogen.

Thin Layer Chromatography

Dissolve sample extract in 200 μL $CHCl_3$. Spot 5 or 10 μL $CHCl_3$ sample solution alongside 2, 4, 8, and 16 μL standard solution. Develop plate 1 h with $CHCl_3$–acetone (9 + 1). Evaporate solvent in fume hood 5 min. Examine plate under longwave UV light to determine presence or absence of aflatoxins. If aflatoxins are present, quantitate by visual comparison of fluorescence intensity of sample with reference standards (1, sec. **26.031**) or by fluorodensitometric measurement.

When densitometer is used to scan sample and aflatoxin reference spots (transmission mode, excitation 365 nm and emission cutoff 430 nm), calculate concentration of B_1 in sample, using the following formula:

$$\text{ng/g} = V_u R_u / V_s R_s W$$

where V_u = sample extract volume; R_u = densitometer response for B_1 in sample aliquot; V_s = μL sample extract spotted; R_s = calculated average densitometer response/ng for 4 B_1 standard spots; W = weight of sample represented by extract (10 g).

Calculate concentrations of B_2, G_1, and G_2 in similar manner.

Confirmation of B_1 by On-Column GC/MS

Divide 20 cm plate into 3 cm channels with pencil. In middle of channels spot amount of sample extracts to contain 60 ng B_1 and one 2 ng B_1 standard. Develop plate with $CHCl_3$–acetone (9 + 1) 1 h. Evaporate solvent. Illuminate standard channel with longwave UV light. Circle standard. Use standard position as guide to locate B_1 in sample extracts. Avoid direct UV irradiation on sample spots. Remove B_1 from TLC plate with Eluchrom, using 2.5 mL $CHCl_3$–acetone–isopropanol (85 + 15 + 5) as eluting solvent. Alternative methods for removing B_1 from TLC plate can be used. Collect eluate in 2 mL concentrator tube. Evaporate solvent in warm water bath (50°C) under stream of nitrogen. Save for GC/MS analysis.

Connect GC capillary column to ion source of mass spectrometer. Set for NICI operating conditions. Calibrate mass scale from m/z 55 to 655 using Fomblin, or FC-43 spiked with dinitrofluorobenzene, trifluoroacetic anhydride, and acetic acid. Adjust instrument parameters to obtain good quality spectrum from 60 ng B_1 standard (in 3 μL acetone) by on-column injection at 40°C. Adjust flow rate to 20 cm/s. Increase column temperature to 250°C in 4 min. Retention time of B_1 is 5–6 min. Dissolve sample extract in 50 μL acetone and concentrate to ca 5 μL. Inject 3 μL on-column and use same GC conditions as for standard. NICI mass spectrum of B_1 consists of m/z 312, 311, and 297 as major ions.

Table 1. Recovery of aflatoxins added to corn and peanut butter

Aflatoxin	Added, ng/g	Found, % Av.	Range	SD (n = 6)	CV, %
		Corn			
B_1	10	82	77–89	4.0	4.8
	5	78	74–82	2.9	3.8
	2	86	80–91	4.1	4.7
B_2	3	87	79–90	4.0	4.6
	1.5	83	79–92	5.0	6.1
	0.6	87	80–93	5.2	6.0
G_1	10	88	83–96	2.5	2.8
	5	81	77–85	3.1	3.8
	2	103	93–111	7.9	7.7
G_2	3	97	85–112	9.3	9.6
	1.5	87	84–91	2.8	3.2
	0.6	127	113–133	11.8	9.2
		Peanut Butter			
B_1	10	76	72–77	2.5	3.2
B_2	3	87	80–98	7.9	9.1
G_1	10	82	80–84	1.6	2.0
G_2	3	117	109–125	6.2	5.3

Results and Discussion

Table 1 gives the average recoveries of aflatoxins added to corn. The recovery of B_1 and G_1 was about 86% at spiking levels of 10, 5, and 2 ng/g. Average recoveries of B_2 and G_2 were 92 and 95%, respectively, at spiking levels of 3 and 1.5 ng/g and 127% for G_2 at 0.6 ng/g.

Table 1 also presents the average recovery values for peanut butter samples spiked with aflatoxins at 10 ng/g (B_1 and G_1) and 3 ng/g (B_2 and G_2) levels. These aflatoxin recoveries have been corrected for aflatoxins present from natural contamination in the blank peanut butter used for spiking (1.1 and 0.4 ng B_1 and B_2/g, respectively, determined by this and the CB method). The average recovery of B_1, B_2, and G_1 was about 82% and that of G_2 was 117%.

Figure 1 shows representative TLC chromatograms. Aflatoxins B_1, B_2, and G_1 were well resolved. A yellow fluorescent tailing interference chromatographed at the same R_f as G_2. This should not be a serious problem because the occurrence of G_2 in corn and peanut butter is much less frequent than that of B_1, B_2, and G_1. According to mycotoxin survey data (G. E. Wood, Food and Drug Administration, Washington, DC, 1983, personal communication) of 548 corn samples analyzed, 67% were contaminated with aflatoxins while only 2% contained G_2; of 146 peanut butter samples analyzed, 19% were contaminated with aflatoxins with 4% containing G_2.

Figure 2 illustrates mass spectra of B_1 isolated from naturally contaminated peanut butter at a level of 20 ng/g and B_1 standard. The signal-to-noise ratio in the spectra is about 75:1. The spectra show a good correlation between sample

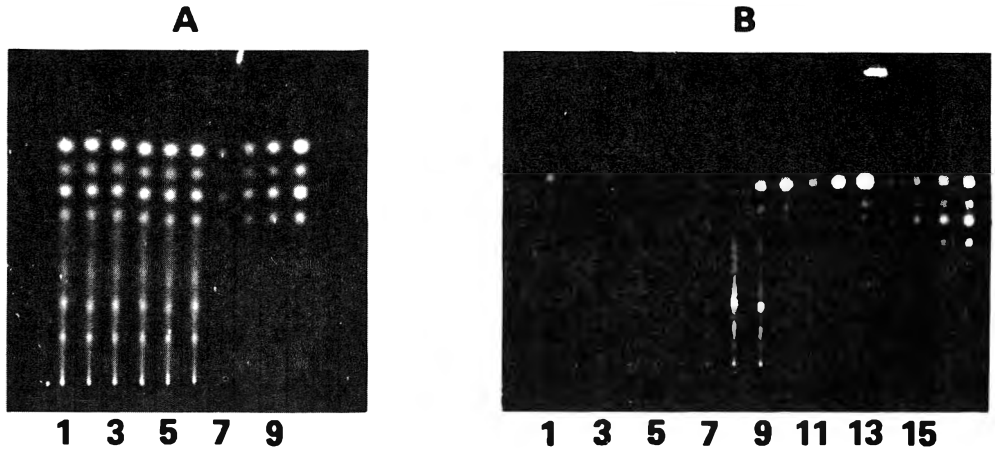

Figure 1. Chromatograms of extracts of peanut butter and corn. TLC plate E. Merck silica gel 60 precoated. Mobile phase $CHCl_3$–acetone (9 + 1), unequilibrated tank.

A, peanut butter: channels 1–6, spiked peanut butter (10 ng B_1 and G_1/g, 3 ng B_2 and G_2/g); 7–10, standards: 2, 4, 8, and 16 μL; B_1 and G_1 0.5 ng/μL; B_2 and G_2 0.15 ng/μL. B, corn: channels 1, 11–13, contaminated corn; 2–7, spiked corn (2 ng B_1 and G_1/g, 0.6 ng B_2 and G_2/g); 8–10, contaminated peanut butter; 14–17, standards: concentrations as in A.

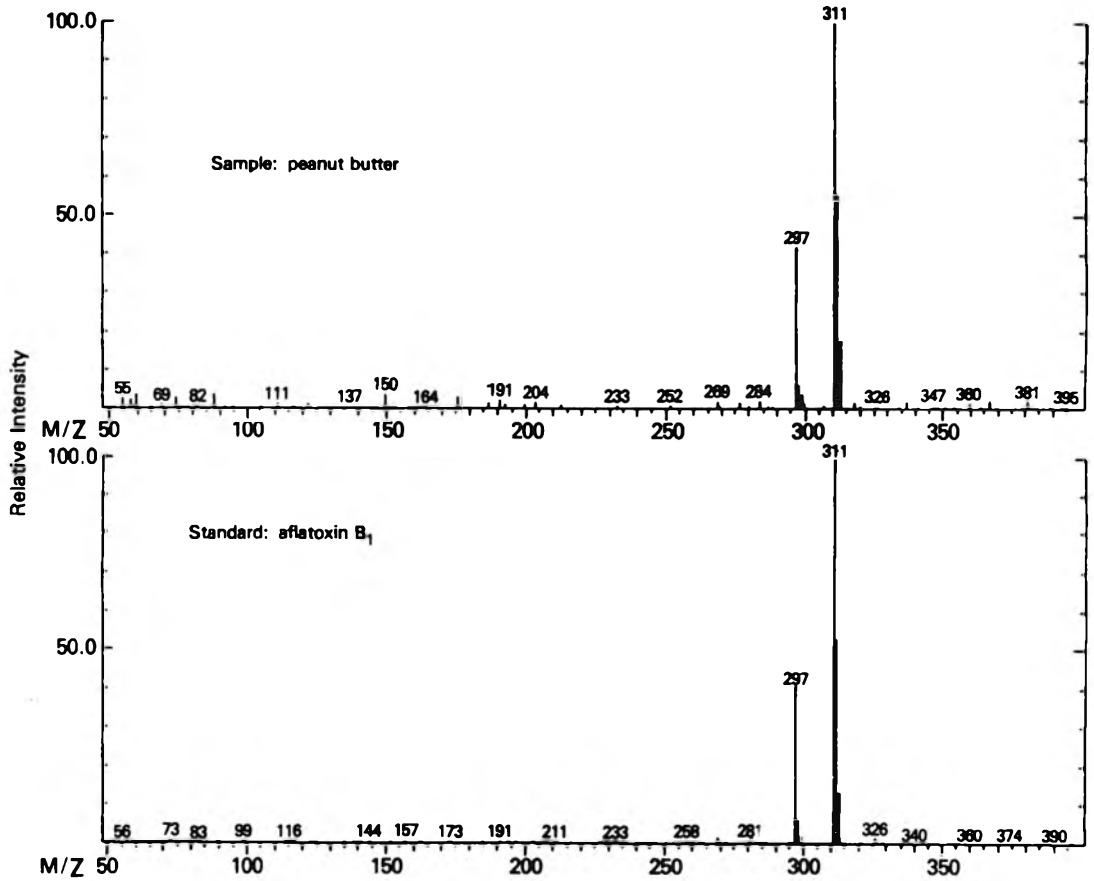

Figure 2. NICI Mass spectra: ca 60 ng B_1 isolated from naturally contaminated peanut butter (20 ng B_1/g); standard, 60 ng B_1.

and standard. The major ions, m/z 312, 311, and 297, were observed in the full scan spectra with no interferences. Spectra of B_1 isolated from naturally contaminated corn were identical to those shown in Figure 2. The advantages of GC introduction over the solid probe introduction are the resolving power of the capillary column and the consequent shorter prior cleanup. This should allow the MS technique to be applied to extracts prepared by many other methods.

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VITAMINS AND OTHER NUTRIENTS

Inter- and Intra-laboratory Variability in Rat Growth Assays for Estimating Protein Quality of Foods

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Protein efficiency ratio (PER), relative PER (RPER), net protein ratio (NPR), relative NPR (RNPR), and nitrogen utilization (NU) methods were investigated in an interlaboratory rat growth study. Six collaborating laboratories studied 7 protein sources (ANRC casein; minced beef; soya assay protein, SAP; pea flour; whole wheat flour, WW; rapeseed protein concentrate, RPC; and egg white solids, EW), and their 10 supplementary or complementary mixtures (casein + Met, SAP + Met, pea flour + Met, WW + Lys, WW + casein, WW + beef, WW + SAP, WW + pea flour, WW + RPC, WW + EW). Test protein(s) were added at the 8% level (N × 6.25). Casein + Met was used as the reference protein. Interlaboratory variation (estimated as between-laboratories coefficients of variation) of PER (up to 17.2%) was greater than that of RPER (up to 14.9%), NU (up to 9.5%), NPR (up to 7.0%), and RNPR, which had the lowest variability (up to 4.7%). In most cases, intralaboratory variation (estimated as within-laboratories coefficients of variation) for all the methods was less than 5%.

Rat growth assays are widely used for evaluating quality of protein in foods, and numerous workers have discussed the merits of these methods (1–11). The protein efficiency ratio (PER) is the official method of evaluating protein quality of foods in Canada and the United States, but this test has been criticized for not properly crediting protein used in maintenance, and for lack of precision, poor reproducibility, and high cost (1–4, 6, 7). The most important disadvantage of the PER test is that PER values of proteins of differing quality are not proportional. Therefore, this method is inappropriate for the calculation of utilizable protein such as in protein rating (grams protein in a reasonable daily intake × PER), which is the official method for regulating protein claims of food labels in Canada (12).

The net protein ratio (NPR, 13), nitrogen utilization (NU, 14), and relative NU (RNU, 14) methods include the protein used for both growth and maintenance and provide protein quality values that are directly proportional to each other within acceptable limits. NPR values, as determined by the original procedures (13), are uncorrected. The scale used for relative NPR (RNPR, NPR of test diet expressed relative to a value of 100 for the NPR of a reference diet), however, is 1 to 100, as it is for net protein utilization (NPU) and biological value (BV). RNPR, which is a modified NPU method (based on body weight), gives results similar to the relative nutritive value (RNV) method of Hegsted and co-workers (15, 16), which is superior to the relative protein value (RPV, 4) method and may be, in theory, the best assay for predicting protein quality by rat growth (1). RNPR is more economical and does not require complex statistical analyses of data as is necessary for the multi-dose assays (17).

As discussed above, rat growth methods for estimating protein quality have been extensively reviewed. Only 3 previous collaborative studies comparing PER and NPR, however, have been reported in the literature (4, 17, 18). The present investigation was undertaken to obtain more information on inter- and intra-laboratory variation in the determination of PER, NPR, relative PER (RPER), RNPR, and NU. Six collaborating laboratories determined these 6 protein quality indices for 7 protein sources (which were recently tested in a collaborative amino acid study, 19), as well as their 10 supplementary or complementary mixtures.

Interlaboratory Study

Diets

Eighteen diets (casein, ANRC; minced beef, freeze dried and defatted; soya assay protein, SAP; pea flour; whole wheat flour, WW; rapeseed protein concentrate, RPC; egg white solids, EW; casein + 0.1% L-Met; SAP + 0.1% L-Met; pea flour + 0.1% L-Met; WW + 0.16% L-lysine; WW + casein; WW + beef; WW + SAP; WW + pea flour; WW + RPC; WW + EW; and N-free) were tested in this investigation.

Protein sources were prepared and analyzed for proximate composition at the organizing laboratory as reported previously by Sarwar et al. (19). These data were used in computing the composition of the experimental diets. Diet formulas (Table 1) and premixes (containing all ingredients except corn oil and corn starch) were given to each collaborator who prepared his own diets by adding required amounts of corn oil and corn starch (Table 1). Each protein source or mixture was added to provide 1.3% dietary nitrogen (or 8% protein, N × 6.25) except for diet 18. For mixtures, each protein source contributed 50% of the dietary protein. The protein source(s) and supplemental amino acids (0.1% L-methionine in diets 8, 9, and 10; 0.2% L-lysine HCl in diet 11) were added at the expense of corn starch. The reference protein was casein with added L-methionine (1.184% of casein or 0.1% of the diet). The diets contained 4.28–4.33 kcal metabolizable energy (ME)/g (ME was calculated by using the Atwater factors 4, 9, and 4 kcal/g for protein, fat, and available carbohydrates, respectively).

Experimental Design

Each laboratory was asked to conduct a randomized complete block design, using 8 blocks of 18 rats. Blocking was by initial body weight, so that rats in the same block had essentially the same initial weight. Eighteen diets were assigned per block. Rats were randomly assigned to cages, and diets within a block were randomly assigned to rats.

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Table 1. Composition (g/6 kg) of diets used in interlaboratory study[a,b]

Diet No.	Casein	Minced beef	SAP[c]	Pea flour	WW[d]	RPC[e]	EW[f]	Cellulose	Corn starch
1	533.9							60.0	4535.6
2		540.3						60.0	4529.2
3			539.6					60.0	4529.9
4				2036.5				19.2	3073.8
5					2840.2			0	2289.3
6						752.9		12.0	4364.6
7							599.0	60.0	4470.5
8	533.9							60.0	4529.6
9			539.6					60.0	4523.9
10				2036.5				19.2	3067.8
11					2840.2			0	2277.3
12	266.9				1420.1			30.0	3412.5
13		270.1			1420.1			30.0	3409.3
14			269.8		1420.1			30.0	3409.6
15				1018.3	1420.1			9.6	2681.5
16					1420.1	376.4		6.0	3327.0
17					1420.1		299.5	30.0	3379.9
18								60.0	5069.5

[a]Each diet contained 60.0, 210.0, 0.5, and 600.0 g vitamins (ref. 20), minerals (ref. 21), zinc oxide, and corn oil, respectively. Diets 8, 9, and 10 also contained 6.0 g supplemental L-methionine, and diet 11 contained 12.0 g supplemental L-lysine HCl.
[b]Diets were prepared by mixing all ingredients except corn oil and corn starch for 45 min in the Ottawa laboratory for distribution to collaborators. Then corn oil and corn starch (obtained locally) were added and all ingredients were mixed for 30 min in each laboratory.
[c]Soya assay protein.
[d]Whole wheat flour.
[e]Rapeseed protein concentrate.
[f]Egg white solids.

Table 2. Protein (N × 6.25) contents of diets (%) for 6 participating laboratories[a]

Diet[b]	1	2	3	4	5	6	Mean ± SEM
Casein	8.43	9.31	8.69	8.51	8.33	7.90	8.53 ± 0.19
Minced beef	8.15	8.53	8.88	8.40	8.50	7.60	8.34 ± 0.18
SAP	8.15	8.70	8.81	8.49	8.69	8.00	8.47 ± 0.13
Pea flour	8.33	8.55	8.51	8.42	8.28	8.20	8.38 ± 0.06
WW	8.34	8.69	8.13	8.35	8.46	8.00	8.33 ± 0.10
RPC	8.24	8.59	8.94	8.21	8.41	7.90	8.38 ± 0.15
EW	8.34	8.50	8.56	8.19	8.28	8.00	8.31 ± 0.08
Casein + Met	8.24	8.66	8.75	8.61	8.38	8.00	8.44 ± 0.12
SAP − Met	8.34	9.05	9.13	8.33	8.42	7.90	8.53 ± 0.19
Pea flour + Met	8.43	8.35	8.63	8.16	8.39	8.20	8.36 ± 0.07
WW + Lys	8.61	8.66	8.44	8.51	8.56	8.00	8.46 ± 0.10
WW + casein	8.24	8.79	8.69	8.66	8.90	8.60	8.65 ± 0.09
WW + minced beef	8.15	8.62	8.94	8.40	8.44	7.60	8.36 ± 0.19
WW − SAP	8.24	8.68	9.25	8.22	8.33	7.70	8.40 ± 0.21
WW − pea flour	8.15	8.40	8.88	8.27	8.25	7.70	8.28 ± 0.16
WW − RPC	8.15	8.66	8.94	8.18	8.33	8.00	8.38 ± 0.15
WW − EW	8.33	8.66	8.75	8.20	8.22	7.80	8.33 ± 0.14

[a]Diets were mixed and analyzed at each participating laboratory.
[b]Abbreviations for protein sources are defined in Table 1.

Collaborators used 21–23-day-old male weanling rats. Laboratories 1, 2, and 6 used CD Sprague Dawley rats from the same supplier (Charles River Canada Inc., St. Constant, Québec). Laboratories 3 and 4 obtained Sprague Dawley-derived rats from Simonsen Laboratories Inc., Gilroy, CA; and Harlan Industries, Indianapolis, IN, respectively. Laboratory 5 used CD Sprague Dawley rats supplied by Charles River Breeding Colony, Wilmington, MA. Rats were kept in stock cages and fed a commercial rat chow diet (ground) for 2 days. Rats were then distributed into groups of 8 having approximately the same mean weights.

Animals were housed in individual screen-bottom cages at 24–25°C and 40–50% humidity. Food and water were provided ad libitum for 4 weeks (2 weeks for the N-free diet), during which time food consumption and weight gains were recorded. All indices studied were calculated from these data.

Calculations of Protein Quality Indices

PER, RPER, NPR, RNPR, and NU calculations were made from the data from the same animals fed the test diets for either 2 weeks (NPR, RNPR, and NU) or 4 weeks (PER and RPER). Investigators analyzed their diets for nitrogen, and these values were used in calculating protein (N × 6.25) intake. The 5 protein quality indices were calculated within each block. For each laboratory, protein quality indices for each diet were calculated by averaging within-block values over all blocks. PER, RPER, NPR, RNPR, and NU were calculated according to the following formulas:

$$\text{PER} = \frac{\text{weight gain of test animal}}{\text{protein consumed by test animal}}$$

$$\text{RPER} = \frac{\text{PER of test animal}}{\text{PER of reference animal}} \times 100$$

$$\text{NPR} = \frac{\text{weight gain of test animal} + \text{weight loss of nonprotein animal}}{\text{protein consumed by test animal}}$$

$$\text{RNPR} = \frac{\text{NPR of test animal}}{\text{NPR of reference animal}} \times 100$$

$$\text{NU} = \frac{\text{weight gain of test animal} + 0.1\,(\text{initial} + \text{final weight})}{\text{protein consumed by test animal}}$$

The maintenance factor, 0.1 (initial + final weight), used in calculating NU is usually similar in magnitude to the weight loss of the nonprotein group included in the NPR method. To facilitate comparability to NPR, the formula for NU was

Table 3. PER values (± SEM) for 6 participating laboratories[a]

Diet[b]	1	2	3	4	5	6
Casein	3.35 ± 0.07	2.86 ± 0.09	3.04 ± 0.07	2.91 ± 0.10	3.36 ± 0.10	3.27 ± 0.16
Minced beef	3.66 ± 0.06	3.38 ± 0.12	3.21 ± 0.08	3.02 ± 0.04	3.50 ± 0.10	3.39 ± 0.13
SAP	1.92 ± 0.09	1.53 ± 0.18	1.54 ± 0.07	1.52 ± 0.06	1.66 ± 0.06	1.26 ± 0.02
Pea flour	1.98 ± 0.05	1.43 ± 0.08	1.77 ± 0.06	1.12 ± 0.06	1.64 ± 0.10	1.30 ± 0.07
WW	1.13 ± 0.06	0.82 ± 0.04	0.92 ± 0.09	0.95 ± 0.09	1.23 ± 0.11	0.71 ± 0.04
RPC	3.59 ± 0.07	3.32 ± 0.15	3.19 ± 0.06	2.95 ± 0.07	3.42 ± 0.13	3.28 ± 0.10
EW	3.97 ± 0.05	3.61 ± 0.12	3.65 ± 0.06	3.44 ± 0.09	3.85 ± 0.15	3.82 ± 0.08
Casein + Met	4.29 ± 0.05	4.10 ± 0.13	3.99 ± 0.05	3.49 ± 0.11	4.32 ± 0.04	4.07 ± 0.09
SAP + Met	2.83 ± 0.04	2.71 ± 0.10	2.60 ± 0.08	2.31 ± 0.14	2.72 ± 0.05	2.13 ± 0.04
Pea flour + Met	3.26 ± 0.06	3.10 ± 0.09	2.79 ± 0.11	2.60 ± 0.10	3.05 ± 0.04	2.79 ± 0.11
WW + Lys	2.16 ± 0.06	1.55 ± 0.12	1.59 ± 0.05	1.59 ± 0.08	1.95 ± 0.10	1.39 ± 0.10
WW + casein	3.44 ± 0.06	2.88 ± 0.16	3.04 ± 0.07	2.60 ± 0.06	3.21 ± 0.08	2.97 ± 0.13
WW + minced beef	3.29 ± 0.07	2.96 ± 0.11	2.90 ± 0.06	2.58 ± 0.06	3.08 ± 0.07	3.12 ± 0.13
WW + SAP	2.46 ± 0.06	2.14 ± 0.13	2.05 ± 0.06	2.06 ± 0.05	2.41 ± 0.08	2.11 ± 0.12
WW + pea flour	2.62 ± 0.07	1.66 ± 0.16	2.41 ± 0.07	2.05 ± 0.07	2.29 ± 0.10	1.94 ± 0.09
WW + RPC	2.44 ± 0.07	2.01 ± 0.16	2.14 ± 0.06	1.95 ± 0.06	2.35 ± 0.11	1.80 ± 0.08
WW + EW	3.16 ± 0.07	2.84 ± 0.10	2.72 ± 0.07	2.71 ± 0.08	3.21 ± 0.08	2.67 ± 0.11

[a]N = 8 for laboratories 1, 2, 4, 5; 9 for laboratory 3; 7 for laboratory 6.
[b]Abbreviations for protein sources are defined in Table 1.

Table 4. RPER values (± SEM) for 6 participating laboratories[a]

Diet[b]	1	2	3	4	5	6
Casein	70 ± 1	70 ± 2	76 ± 2	83 ± 2	78 ± 2	80 ± 2
Minced beef	85 ± 1	82 ± 1	81 ± 2	87 ± 2	81 ± 2	83 ± 3
SAP	45 ± 2	37 ± 3	38 ± 2	45 ± 1	38 ± 1	31 ± 1
Pea flour	47 ± 1	35 ± 2	44 ± 2	33 ± 1	38 ± 2	32 ± 1
WW	26 ± 1	20 ± 1	23 ± 2	27 ± 2	28 ± 2	17 ± 1
RPC	83 ± 2	81 ± 2	80 ± 2	85 ± 2	79 ± 2	81 ± 1
EW	91 ± 1	88 ± 2	90 ± 2	94 ± 2	89 ± 3	94 ± 2
Casein + Met	100	100	100	100	100	100
SAP + Met	66 ± 1	67 ± 4	66 ± 2	66 ± 2	63 ± 1	53 ± 1
Pea flour + Met	76 ± 1	73 ± 2	70 ± 3	74 ± 2	71 ± 1	69 ± 2
WW + Lys	50 ± 1	38 ± 2	40 ± 1	46 ± 1	45 ± 2	34 ± 2
WW + casein	80 ± 1	70 ± 2	76 ± 2	74 ± 2	74 ± 2	72 ± 2
WW + minced beef	77 ± 1	72 ± 2	73 ± 1	74 ± 1	71 ± 2	76 ± 2
WW + SAP	57 ± 1	52 ± 2	51 ± 1	59 ± 1	56 ± 2	52 ± 2
WW + pea flour	61 ± 1	40 ± 3	60 ± 1	59 ± 2	53 ± 2	48 ± 2
WW + RPC	57 ± 1	49 ± 3	54 ± 2	56 ± 2	54 ± 2	44 ± 1
WW + EW	73 ± 1	70 ± 2	68 ± 2	78 ± 1	74 ± 2	66 ± 3

[a]N = 8 for laboratories 1, 2, 4, 5; 9 for laboratory 3; 7 for laboratory 6.
[b]Abbreviations for protein sources are defined in Table 1.

changed from the original definition (14) which is based on nitrogen consumed rather than protein consumed.

Statistical Methods

Analysis of variance was performed on PER, RPER, NPR, RNPR, and NU data. Estimates of reproducibility (interlaboratory variability) and repeatability (intralaboratory variability) were obtained by the Youden and Steiner method (22). Tests of significance were conducted at $\alpha = 0.01$ unless otherwise specified. In all cases, it was necessary to partition the diets into 3 groups to circumvent significant diet by laboratory interactions (i.e., analyzing the 3 groups separately). Coefficients of variation (CV) were calculated according to the following formulas:

Within-laboratories CV
= (repeatability standard deviation/mean) × 100

Between-laboratories CV
= (reproducibility standard deviation/mean) × 100

Results and Discussion

Table 2 gives protein contents of the diets, as determined by each collaborator In general, laboratory 6 had lower estimates whereas laboratories 2 and 3 appeared to have higher estimates than the average. However, the interlaboratory variation (estimated as between-laboratories CV) for the determination of protein was less than 3% in all cases.

Individual laboratory data for PER, RPER, NPR, RNPR, and NU are shown in Tables 3, 4, 5, 6, and 7, respectively.

In most cases, all 5 protein quality methods ranked the diets in the same order (Table 8), and positive correlations among the protein quality indices were highly significant (r = 0.98–1.00). In all diets, the NU values were similar to NPR values (Table 8). Unlike the PER method, the NPR and NU methods credit protein used for both growth and maintenance. Therefore, the NPR or NU values of all diets were higher than their PER values (Table 8). The RNPR values of all diets were higher than their RPER values. These differences were especially marked in the case of diets containing poor quality protein such as wheat flour.

Before conducting analyses of variance, the data (Tables 3–7) were edited to obtain statistical balance. This editing was required because laboratory 6 produced results for only 7 blocks. From each of laboratories 1, 2, 4, and 5, one block of results was removed. Two blocks were removed from laboratory 3 (results were produced for 9 blocks). In each case, the block removed was selected so that the blocks remaining were as alike as possible with respect to mean initial body weight. Hence, blocks with atypically high or low initial body weights were deleted to minimize differences among laboratories attributable to initial body weight extremes.

The initial analyses of variance showed highly significant laboratory by diet interactions for all indices ($P < 0.001$ for PER, RPER, RNPR, and NU, and $P = 0.0023$ for NPR). Similar highly significant interactions for PER and NPR have been reported (23–25). Possible causes of these interactions in the present study are: *(1)* Diet preparation: Each laboratory prepared its own set of diets, mixing some centrally distrib-

Table 5. NPR values (± SEM) for 6 participating laboratories[a]

Diet[b]	1	2	3	4	5	6
Casein	4.60 ± 0.09	4.53 ± 0.16	4.70 ± 0.11	4.08 ± 0.10	4.78 ± 0.08	4.75 ± 0.23
Minced beef	5.08 ± 0.07	4.78 ± 0.13	4.69 ± 0.13	4.46 ± 0.10	4.78 ± 0.08	5.11 ± 0.09
SAP	2.96 ± 0.15	2.63 ± 0.12	2.63 ± 0.07	2.76 ± 0.12	2.83 ± 0.09	2.72 ± 0.06
Pea flour	3.10 ± 0.10	2.95 ± 0.17	2.94 ± 0.09	2.62 ± 0.06	2.84 ± 0.17	2.92 ± 0.12
WW	2.40 ± 0.04	2.17 ± 0.08	2.36 ± 0.07	2.23 ± 0.11	2.44 ± 0.09	2.52 ± 0.15
RPC	4.76 ± 0.07	4.71 ± 0.13	4.37 ± 0.11	4.28 ± 0.13	4.61 ± 0.07	4.89 ± 0.15
EW	5.20 ± 0.06	5.09 ± 0.14	5.05 ± 0.08	4.67 ± 0.11	5.28 ± 0.10	5.31 ± 0.12
Casein + Met	5.34 ± 0.08	5.36 ± 0.12	5.20 ± 0.13	4.84 ± 0.10	5.64 ± 0.09	5.60 ± 1.0
SAP + Met	3.97 ± 0.10	3.72 ± 0.15	3.49 ± 0.08	3.34 ± 0.12	3.89 ± 0.21	3.79 ± 0.12
Pea flour + Met	4.51 ± 0.06	4.56 ± 0.15	3.98 ± 0.20	4.01 ± 0.13	4.38 ± 0.06	4.66 ± 0.13
WW + Lys	3.05 ± 0.08	3.02 ± 0.14	2.74 ± 0.05	2.60 ± 0.15	3.15 ± 0.17	2.94 ± 0.10
WW + casein	4.56 ± 0.08	4.18 ± 0.22	4.17 ± 0.14	3.70 ± 0.10	4.32 ± 0.09	4.37 ± 0.11
WW + minced beef	4.47 ± 0.09	4.21 ± 0.14	4.06 ± 0.09	3.67 ± 0.07	4.23 ± 0.05	4.50 ± 0.23
WW + SAP	3.48 ± 0.09	3.31 ± 0.16	3.12 ± 0.08	3.08 ± 0.13	3.58 ± 0.15	3.74 ± 0.18
WW + pea flour	3.88 ± 0.05	3.30 ± 0.06	3.60 ± 0.13	3.22 ± 0.11	3.49 ± 0.09	3.57 ± 0.07
WW + RPC	3.53 ± 0.09	3.19 ± 0.16	3.15 ± 0.13	3.28 ± 0.08	3.58 ± 0.09	3.51 ± 0.14
WW + EW	4.20 ± 0.08	4.12 ± 0.10	3.70 ± 0.09	4.11 ± 0.11	4.23 ± 0.10	4.26 ± 0.17

[a]N = 8 for laboratories 1, 2, 4, 5; 9 for laboratory 3; 7 for laboratory 6.
[b]Abbreviations for protein sources are defined in Table 1.

Table 6. RNPR values (± SEM) for 6 participating laboratories[a]

Diet[b]	1	2	3	4	5	6
Casein	86 ± 1	85 ± 2	90 ± 2	85 ± 1	85 ± 2	85 ± 3
Minced beef	95 ± 1	89 ± 2	90 ± 2	93 ± 1	85 ± 1	91 ± 2
SAP	55 ± 2	49 ± 2	50 ± 1	56 ± 2	50 ± 2	49 ± 1
Pea flour	58 ± 1	55 ± 2	56 ± 2	54 ± 1	50 ± 2	52 ± 2
WW	46 ± 1	41 ± 2	45 ± 2	46 ± 1	43 ± 1	45 ± 2
RPC	90 ± 2	88 ± 2	84 ± 2	89 ± 1	81 ± 1	87 ± 2
EW	96 ± 1	95 ± 1	97 ± 2	96 ± 2	94 ± 1	95 ± 2
Casein + Met	100	100	100	100	100	100
SAP + Met	74 ± 2	70 ± 3	68 ± 2	70 ± 1	67 ± 2	69 ± 1
Pea flour + Met	85 ± 1	85 ± 2	76 ± 2	82 ± 2	78 ± 1	83 ± 2
WW + Lys	58 ± 1	56 ± 2	53 ± 1	55 ± 1	56 ± 3	53 ± 3
WW + casein	84 ± 1	78 ± 2	80 ± 1	76 ± 1	76 ± 1	78 ± 1
WW + minced beef	84 ± 1	78 ± 2	78 ± 2	76 ± 1	75 ± 1	80 ± 3
WW + SAP	65 ± 1	63 ± 2	60 ± 1	64 ± 2	64 ± 2	67 ± 2
WW + pea flour	72 ± 1	61 ± 1	69 ± 2	67 ± 2	62 ± 1	64 ± 2
WW + RPC	66 ± 1	59 ± 2	60 ± 2	68 ± 2	64 ± 2	63 ± 2
WW + EW	79 ± 1	77 ± 1	72 ± 2	85 ± 2	75 ± 1	76 ± 2

[a]N = 8 for laboratories 1, 2, 4, 5; 9 for laboratory 3; 7 for laboratory 6.
[b]Abbreviations for protein sources are defined in Table 1.

uted key ingredients with other ingredients (such as corn oil and corn starch) obtained locally. Variations in locally obtained ingredients together with variations in mixing may be responsible for diet by laboratory interactions. *(2)* Experimental error: The true experimental error could not be estimated because the experiment was not replicated within laboratories. The protein quality indices being studied are traditionally measured from a group (or groups) of rats. It is clear that none of these indices would normally be based on individual block responses. However, to obtain some measure of within-laboratory variability, it was necessary to use individual block responses. This lack of true replication fundamentally results in an underestimate of the experimental error and may result in an underestimate of repeatability. This may increase the chances of a false declaration of significant interactions. *(3)* Nitrogen analyses: Each laboratory analyzed nitrogen content of each diet. Variations in these analyses could result in significant interactions. *(4)* Protein quality: From a visual inspection of graphs showing each laboratory's response over all diets, it was apparent that the responses followed 3 general patterns. These patterns correspond to diets of "high" protein quality (casein, minced beef, RPC, EW, casein + Met, pea flour + Met, WW + casein, WW + minced beef, and WW + EW diets), "medium" protein quality (SAP + Met, WW + SAP, WW + pea flour, and WW + RPC diets), and "low" protein quality (SAP, pea flour, WW, and WW + Lys diets). Further investigation of these patterns was done by repeating analyses of variance on each subgroup of diets. Statistical tests generally confirmed the absence of interactions.

Tables 9, 10, and 11 give estimates of repeatability and reproducibility and coefficients of variation (CV) for the high, medium, and poor protein quality diets respectively. These estimates apply to responses which have been averaged over 8 individual values. Repeatability estimates may be seriously underestimated because true within-laboratory replication did not exist in the experimental design. In general, reproducibility estimates were about twice as large as those for repeatability.

In high quality diets, the between-laboratories CV values for RNPR (3.9%) and RPER (4.2%) were lower than those for other protein quality indices (6.1–7.7%, Table 9). Within-laboratories CV values for all the indices were less than 3% (2.0–2.8%, Table 9).

In medium quality diets, the between-laboratories CV values for PER (11.1%) and RPER (10.2%) were higher than those for other protein quality indices (4.5–7.5%, Table 10). The within-laboratories CV values for all the protein quality indices were not more than 4% (2.7–4.0%, Table 10). RNPR had the lowest between- and within-laboratories CV values.

In poor quality diets, the between-laboratories CV values for PER and RPER were about 3 times higher than those for RNPR and NPR, while NU occupied an intermediate position in terms of between-laboratories CV (Table 11). Within-laboratories CV values for PER and RPER were also higher than for other protein quality indices.

Table 7. NU values (± SEM) for 6 participating laboratories[a]

Diet[b]	1	2	3	4	5	6
Casein	4.89 ± 0.10	4.94 ± 0.17	5.08 ± 0.10	4.21 ± 0.13	5.13 ± 0.16	4.83 ± 0.24
Minced beef	5.47 ± 0.08	5.22 ± 0.15	5.08 ± 0.12	4.58 ± 0.10	5.14 ± 0.10	5.32 ± 0.09
SAP	3.05 ± 0.16	2.85 ± 0.14	2.80 ± 0.07	2.70 ± 0.11	2.96 ± 0.08	2.43 ± 0.09
Pea flour	3.13 ± 0.10	3.21 ± 0.12	3.12 ± 0.07	2.44 ± 0.08	2.88 ± 0.14	2.56 ± 0.15
WW	2.34 ± 0.08	2.28 ± 0.08	2.52 ± 0.07	2.01 ± 0.14	2.50 ± 0.10	2.18 ± 0.11
RPC	5.10 ± 0.09	5.10 ± 0.12	4.75 ± 0.10	4.40 ± 0.15	4.98 ± 0.08	5.10 ± 0.17
EW	5.60 ± 0.06	5.54 ± 0.14	5.49 ± 0.06	4.87 ± 0.13	5.68 ± 0.09	5.55 ± 0.10
Casein + Met	5.74 ± 0.07	5.85 ± 0.16	5.65 ± 0.13	4.92 ± 0.15	6.09 ± 0.10	5.87 ± 0.11
SAP + Met	4.25 ± 0.10	4.04 ± 0.16	3.69 ± 0.06	3.34 ± 0.17	3.96 ± 0.17	3.77 ± 0.10
Pea flour + Met	4.82 ± 0.05	4.96 ± 0.09	4.28 ± 0.20	4.10 ± 0.17	4.71 ± 0.05	4.72 ± 0.12
WW + Lys	3.13 ± 0.10	3.29 ± 0.10	2.88 ± 0.06	2.55 ± 0.07	3.31 ± 0.15	2.77 ± 0.17
WW + casein	4.90 ± 0.08	4.41 ± 0.26	4.50 ± 0.12	3.79 ± 0.10	4.64 ± 0.10	4.51 ± 0.14
WW + minced beef	4.80 ± 0.09	4.64 ± 0.17	4.39 ± 0.09	3.71 ± 0.08	4.53 ± 0.03	4.54 ± 0.21
WW + SAP	3.67 ± 0.09	3.61 ± 0.20	3.33 ± 0.08	3.08 ± 0.18	3.82 ± 0.13	3.71 ± 0.20
WW + pea flour	3.97 ± 0.12	3.43 ± 0.13	3.84 ± 0.11	3.21 ± 0.12	3.68 ± 0.12	3.40 ± 0.07
WW + RPC	3.71 ± 0.10	3.47 ± 0.19	3.39 ± 0.11	3.30 ± 0.07	3.78 ± 0.13	3.44 ± 0.14
WW + EW	4.49 ± 0.11	4.49 ± 0.11	4.11 ± 0.08	4.21 ± 0.12	4.53 ± 0.13	4.28 ± 0.17

[a]*N* = 8 for laboratories 1, 2, 4, 5; 9 for laboratory 3; 7 for laboratory 6.
[b]Abbreviations for protein sources are defined in Table 1.

Table 8. Ranking of diets by different protein quality indices[a]

Diet[b]	PER	NPR	NU	RPER	RNPR
Casein + Met	4.04	5.30	5.68	100	100
Egg white solids	3.71	5.08	5.43	91	95
Minced beef	3.36	4.83	5.16	83	91
RPC	3.29	4.59	4.90	81	87
Casein	3.13	4.55	4.83	78	86
Pea flour + Met	2.91	4.33	4.59	72	81
WW + casein	3.01	4.20	4.47	74	79
WW + beef	2.97	4.16	4.41	73	79
WW + EW	2.90	4.09	4.36	72	77
SAP + Met	2.55	3.72	3.87	64	70
WW + pea flour	2.18	3.50	3.60	54	66
WW + SAP	2.21	3.37	3.53	55	64
WW + RPC	2.08	3.34	3.49	51	63
WW + Lys	1.70	2.91	2.98	42	55
Pea flour	1.56	2.88	2.89	39	54
SAP	1.60	2.74	2.79	39	51
WW	0.95	2.35	2.29	23	44

[a]Mean of 6 collaborating laboratories.
[b]Abbreviations for protein sources are defined in Table 1.

Table 9. Estimates of repeatability and reproducibility and coefficients of variation for the "high" protein quality diets[a]

				CV, %[d]	
Index	Mean	Repeatability[b]	Reproducibility[c]	Within-labs	Between-labs
PER	3.26	0.09	0.25	2.8	7.7
RPER	78.00	1.92	3.29	2.5	4.2
NPR	4.57	0.12	0.28	2.6	6.1
RNPR	84.00	1.67	3.26	2.0	3.9
NU	4.87	0.13	0.33	2.7	6.8

[a]Refers to diets with protein quality similar to diets 1, 2, 6, 7, 8, 10, 12, 13, and 17 of this experiment.
[b]Repeatability estimates the standard deviation applicable to the average of 8 individual responses of a randomly chosen diet with a selected laboratory.
[c]Reproducibility estimates the standard deviation applicable to the average of 8 individual responses of a randomly chosen diet analyzed by a randomly chosen laboratory.
[d]Within-laboratories CV [(repeatability standard deviation/mean) × 100] values are applicable to the average of 8 individual responses of a randomly chosen diet within a selected laboratory.
Between-laboratories CV [(reproducibility standard deviation/mean) × 100] values are applicable to the average of 8 individual responses of a randomly chosen diet analyzed by a randomly chosen laboratory.

In general, RNPR and NPR seemed to be the least variable over laboratories (Tables 9–11). PER and RPER varied the most across laboratories, and the variation increased as protein quality decreased.

The lower precision of the PER method than the NPR or RNPR method noted in this investigation was in agreement with a recent report (17). McLaughlan et al. (17) reported that PER values of casein + Met, egg white, wheat gluten, SAP, and wheat gluten + SAP diets were more variable (CV = 16.1–54.3%) than their NPR values (CV = 10.4–16.8%) which varied more than RNPR (CV = 7.6–13.0%) or RNU (CV = 7.6–13.6%) values. McLaughlan et al. (17) had recommended the use of ANRC casein or casein + Met as the reference diet in protein quality evaluations. In our study, casein + Met diet (which was used as the standard) gave the highest protein quality results and appeared to be of uniform nutritional quality (Tables 3–7).

Hackler et al. (24) and Happich et al. (25) have recently reported the results of the AACC/ASTM collaborative study on PER and NPR, respectively. Standard deviations for

Table 10. Estimates of repeatability and reproducibility and coefficients of variation for the "medium" protein quality diets[a]

Index	Mean	Repeatability[b]	Reproducibility[c]	CV, %[d] Within-labs	CV, %[d] Between-labs
PER	2.26	0.09	0.25	4.0	11.1
RPER	56.00	2.00	5.72	3.6	10.2
NPR	3.48	0.12	0.24	3.4	7.0
RNPR	66.00	1.77	2.93	2.7	4.5
NU	3.62	0.13	0.27	3.6	7.5

[a]Refers to diets with protein quality similar to diets 9, 14, 15, and 16 of this experiment.
[b–d]See footnotes *b–d*, Table 9.

Table 11. Estimates of repeatability and reproducibility and coefficients of variation for the "poor" protein quality diets[a]

Index	Mean	Repeatability[b]	Reproducibility[c]	CV, %[d] Within-labs	CV, %[d] Between-labs
PER	1.45	0.08	0.25	5.5	17.2
RPER	36.00	1.87	5.37	5.2	14.9
NPR	2.72	0.11	0.14	4.0	5.1
RNPR	51.00	1.64	2.42	3.2	4.7
NU	2.73	0.11	0.26	4.0	9.5

[a]Refers to diets with protein quality similar to diets 3, 4, 5, and 11 of this experiment.
[b–d]See footnotes *b–d*, Table 9.

repeatability and reproducibility (calculated over 7 laboratories, five 10% protein diets, and 3 adaptation periods; 0, 2, and 4 days) for PER (4-week) were 0.16 and 0.30, respectively (24). Adjusting the PER data to casein = 2.5 resulted in improved reproducibility (standard deviation = 0.19). Inter- and intralaboratory variations (measured as CV) for the determination of the 14-day NPR (calculated over 7 laboratories, five 10% protein diets and 3 adaptation periods) were 13.2 and 7.7%, respectively (25). Adjustment of the NPR data to RNPR (ANRC casein = 100) reduced the interlaboratory variation to 9.2% but had little effect on the intralaboratory variation (8.0%). Happich et al. (25) recommended the acceptance of the NPR method (with 5 specifications) as official first action as an alternative method to the PER assay. The specifications for the NPR method were *(1)* 1.6% dietary nitrogen; *(2)* 14-day feeding periods; *(3)* 2-day adaptation period; *(4)* use of ANRC casein as the reference protein; and *(5)* expression of NPR as RNPR (25).

On the basis of results of this investigation (Tables 8–11) and the study of McLaughlan et al. (17), RNPR is recommended as the most suitable rat growth method for evaluating protein quality of foods. The adoption of the NPR method as an acceptable method for evaluating protein quality of foods for regulatory purposes is under serious consideration by the Canadian Health Protection Branch.

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Quantitative Gas Chromatographic Headspace Determination of Choline in Adult and Infant Formula Products

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A gas chromatographic headspace method has been developed for the determination of choline in liquid and powder formula products. Choline undergoes the Hofmann elimination reaction during a 24 h, 120°C alkaline digestion. Volatile trimethylamine (TMA) is produced and equilibrates between the liquid and vapor phase (headspace). A 50 μL headspace sample is chromatographed on a 28% Pennwalt 223–4% KOH column, with flame ionization detection. Choline is indirectly quantitated by comparing sample TMA peaks with peaks for a reference standard. Ethyl ether is used as an internal standard. Compared with previous methods, this procedure offers improved precision (7.7–9.6% relative standard deviation (RSD)), accuracy, and specificity of both free and bound forms of choline. Estimated analysis rate using manual injection is 160 samples/week, requiring 35 work-hours. Automation of this procedure, using a commercially available headspace analyzer, resulted in a further improvement in precision (1.60–6.91% RSD) and a throughput of 400 samples/week.

Choline, (β-hydroxyethyl)trimethylammonium hydroxide (Figure 1), appears to be universally synthesized in the animal body. It is, however, considered a dietary essential because the demand for choline can surpass the body's capacity for de novo synthesis. As described by Wilson and Lorenz (1), 3 functions for choline in the body have been established: Choline is a substrate for the synthesis of the neurotransmitter acetylcholine, it is a source of labile methyl groups, and it is an essential component of phospholipids. As a component of phospholipids, choline plays a crucial role in maintaining cellular membrane structure and cellular permeability, and in transporting fat in the bloodstream.

In formula products, choline exists in free and/or bound (lecithin phosphatidyl choline) forms and is chemically designated a quaternary amine. The most widely used method for the quantitative determination of both forms involves multiple extraction and hydrolysis steps designed to isolate choline from the sample matrix, followed by analyte complexation with Reineckate reagent, and quantitation by spectrophotometry (2). This time-consuming method lacks precision, specificity, and accuracy, and often requires numerous repeat analyses to achieve reproducible results.

This report describes a simple, relatively fast method for determining both free and bound choline in a complex matrix, using a gas chromatography (GC)-headspace technique. The procedure requires an initial 24 h, 120°C alkaline digestion of formula in a sealed glass vial, as described by Garavelli (3). During digestion, choline undergoes the Hofmann elimination reaction shown in Figure 2 (4). On completion, the quaternary ammonium ion is cleaved from choline with the production of 1 mole of volatile trimethylamine (TMA) for each mole of reactant choline.

The quantitatively produced TMA equilibrates between the liquid and vapor phases (headspace) (5, 6). When equilibration is complete, the gaseous headspace is sampled and analyzed for TMA by gas chromatography, with final TMA detection by flame ionization.

To eliminate possible sample matrix interferences, a standard addition procedure is used for quantitation (5). The choline concentration is determined indirectly by simple comparison of sample-produced TMA against TMA produced from a choline chloride-spiked reference standard. Ethyl ether is used as an internal standard.

The chromatographic system was automated by using a commercially available headspace analyzer, essentially a gas chromatograph fitted with a headspace autosampler. Minor modifications were made in the procedure to study the use of the automated method for analysis of other formula products, including powders.

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METHOD

Manual Injection

Apparatus

(a) *Headspace sample vials.*—30 mL glass vials No. 0105-0129 (Perkin Elmer, Norwalk, CT 06856).

(b) *Septa.*—Gray butyl, No. 13050 (Pierce Chemical Co., Rockford, IL 61105) or equivalent.

(c) *Seals.*—Aluminum 20 mm, No. 13214 (Pierce Chemical Co.) or equivalent.

(d) *Syringe.*—100 μL pressure-lock series gas syringe (Precision Sampling Corp., Baton Rouge, LA 70815) or equivalent.

(e) *Forced air oven.*—Isotemp oven, Series 400 (Fisher Scientific Co., Pittsburgh, PA 15219) or equivalent.

(f) *Gas chromatograph.*—Hewlett-Packard Model 5840 with flame ionization detector (Hewlett-Packard, Avondale, PA 19311) or equivalent. Operating conditions: temperatures (°C)—injection port 150; oven (isothermal) 60; detector 200 Gas flows (mL/min)—helium carrier 25; hydrogen 30; air 200.

(g) *Chromatographic column.*—Coiled 6 ft × 2 mm id glass, packed with 28% Pennwalt 223 + 4% KOH on 80–100 mesh Gas-Chrom R (Applied Science Laboratories, Inc., State College, PA 16801) or equivalent.

Reagents

(a) *Choline chloride.*—99+% Gold Label, 23,994-1 (Aldrich Chemical Co., PO Box 355, Milwaukee, WI 53201) or equivalent.

(b) *Choline chloride stock solution.*—11.5 or 10.0 mg/mL of choline free base. Dry ca 1 g choline chloride in a vacuum oven at 60°C for 12 h, and then store in a desiccator. Accurately weigh, as quickly as possible, ca 576 mg of the previously dried choline chloride into a preweighed beaker. (Note: The choline chloride reference standard is hygroscopic and must be weighed within 5 min after removing from desiccator.) Quantitatively transfer weighed sample with water into a 50 mL volumetric flask and dilute to volume with water. Calculate exact weight of choline free base by following equation:

$$\text{Choline, mg/mL} = \frac{[\text{mg choline chloride} \times 121.18\ (\text{MW choline})]}{[139.63\ (\text{MW choline chloride}) \times 50.0\ \text{mL}]}$$

A 576.0 mg choline chloride sample is equivalent to 500 mg choline free base or 10.0 mg/mL. Prepare solution fresh weekly and store in refrigerator.

$$(CH_3)_3\overset{+}{\equiv}N-CH_2-CH_2-OH + OH^-$$

Figure 1. (β-Hydroxyethyl)trimethylammonium hydroxide.

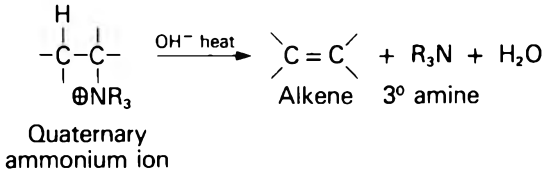

Figure 2. Hofmann elimination reaction.

(c) *Ethyl ether internal standard.*—By syringe, add 0.3 mL ethyl ether to 5 mL water in a 10 mL volumetric flask. Dilute mixture to volume with water. Prepare fresh daily.

Preparation of Sample, Standard Addition Reference, and Blank

Sample.—Mix sample thoroughly by shaking. Let bubbles dissipate and by syringe add 10 mL sample to 30 mL glass vial containing 5 ± 0.5 g KOH. Using microsyringe, add 6 μL prepared internal standard to the liquid, with syringe needle submerged. Cap vial with septum, seal immediately, and shake vial until KOH dissolves. Place vial in 120°C air oven and let solution digest 24 h. After digestion, let samples cool and equilibrate ≥ 2 h.

Standard addition reference.—Using microsyringe, add 150 μL choline chloride stock solution to 3 additional vials containing 10 mL sample and 5 g KOH. Proceed as for headspace sample, beginning "Using a microsyringe. . . ." Digest standard addition reference simultaneously with sample.

Blank.—Pipet 10 mL water into 30 mL vial containing 5 g KOH. Proceed as for headspace sample, beginning "Using microsyringe. . . ."

Headspace Analysis

Use same headspace analysis procedure on blank, standard addition references, and samples. After cooling and equilibration, pierce vial septum with the 100 μL gas syringe. Flush syringe once with gaseous headspace, then draw a 50 μL volume. Inject volume into gas chromatograph. Retention times for TMA and ether internal standard are ca 2.40 and 4.70 min, respectively.

System suitability check.—Check system reproducibility by analyzing 10 replicate 50 μL injections of prepared standard addition reference. Relative standard deviation for peak response factors (TMA peak area/internal standard peak area) of 10 injections should be ≤ 5%. Figure 3 shows a typical chromatogram.

Determination of choline.—After completing acceptable system suitability check, make duplicate headspace injections of prepared solutions in following sequence: blank injection, standard addition references, and sample. Additional samples may be prepared and analyzed simultaneously. A set of standard addition references must be prepared for each formula composition to eliminate matrix effects (i.e., Pro-Sobee vs Enfamil). Obtain choline concentration by comparing duplicate injection average peak area response factor (RF) of samples against average RF standard addition references. Perform blank analysis to ensure that no interfering peaks are present in the specific retention windows for TMA and internal standard ethyl ether.

Calculation.—Use averaged RF values to calculate choline concentration as follows:

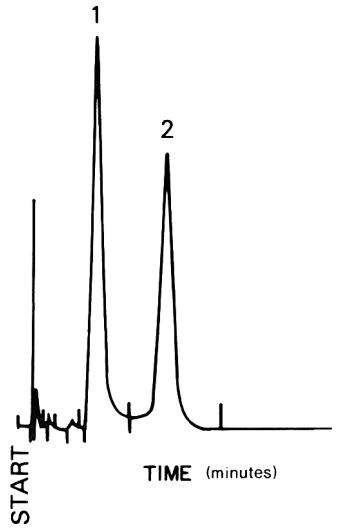

Figure 3. Gas chromatogram of 50 μL headspace sample from Enfamil spiked with 1.5 mg choline/mL: 1, trimethylamine 2.44 min; 2, ethyl ether internal standard 4.72 min.

Choline, mg/qt =

[sample RF/(std addn ref. RF − sample RF)] × 0.15 mg/mL × 947 mL/qt

Modifications for Automated Analysis

Apparatus

Headspace analyzer.—Perkin-Elmer Model F-45 with flame ionization detector.

Procedure

(a) *Liquid samples.*—Add 5 mL sample to 10 mL 50% NaOH (w/v) in 30 mL headspace sample vial. Add 8 μL ethyl ether internal standard solution and 100 μL choline chloride stock solution (for standard addition reference). Carry out digestion and analysis as for manual method. Calculate choline concentration as follows:

Choline, mg/qt =

[sample RF/(std addn ref. RF − sample RF)] × 0.067 mg/mL × 3 × 947

(b) *Powder samples.*—Mix 0.500 g sample with 5 mL water in 30 mL headspace sample vial and add 10 mL 50% NaOH (w/v). Add 8 μL ethyl ether internal standard solution and 100 μL choline chloride stock solution (for standard addition reference). Carry out digestion and analysis as for manual method. Calculate choline concentration as follows:

Choline, mg/g =

{[sample RF/(std addn ref. RF − sample RF)] × 0.067 mg/mL × 15}/0.500 g sample

Results and Discussion

Manual Headspace Analysis

The initial method development program for the determination of choline in liquid infant formula products was designed to demonstrate specificity, linearity of detector response, precision, recovery, and applicability.

Specificity, or the effectiveness of the method to selectively measure an analyte in the presence of potentially interfering substances, was demonstrated by comparing headspace chromatograms of liquid formula digested with KOH and

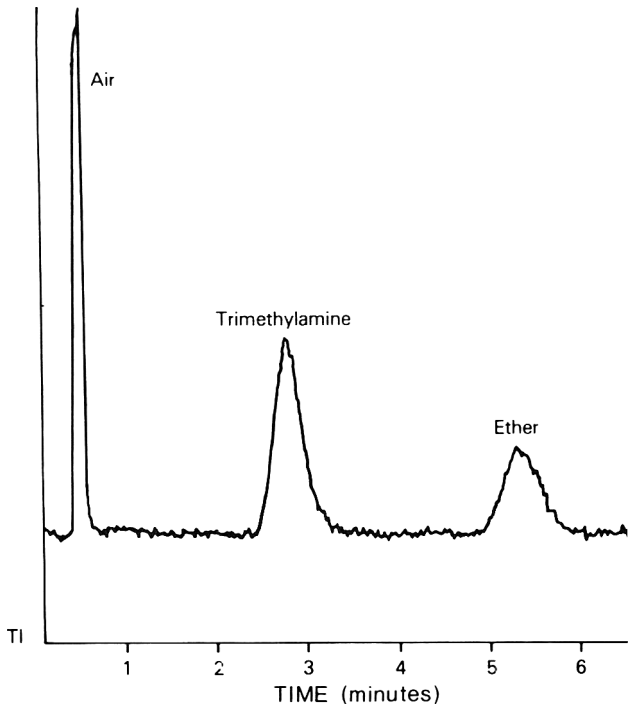

Figure 4. GC/MS total ion chromatogram of Enfamil. Trimethylamine peak at 2.8 min.

without KOH (no TMA produced). No significant interfering peaks were produced by the sample matrix at retention times specific for TMA or ether internal standard. Comparison of samples analyzed with and without the addition of choline chloride showed appropriate spike increases in TMA peak size, again demonstrating specificity.

Specificity was also shown by identifying the TMA produced from a digested Enfamil sample by using gas chromatography/mass spectroscopy (Figures 4 and 5). This was accomplished by comparing mass spectral results on samples against results for a standard solution of 28% TMA–water.

Linearity of detector response was shown by analyzing standard addition Enfamil samples spiked with concentrations of 0.02, 0.05, 0.10, 0.15, and 0.20 mg/mL of choline free base. Linear regression analysis provided a correlation coefficient of 0.993, slope of 7.80/0.01 unit, and y-intercept of 0.994. Prosobee samples spiked at 0.02, 0.05, 0.10, 0.15, 0.20, and 0.25 mg/mL provided a correlation coefficient of 0.993, slope of 3.66/0.01 unit, and y-intercept of 0.57. The minimum detectable level was defined as the lowest level on the linearity graph or 18.9 mg/qt in liquid formula.

Precision studies, designed to determine the ability of the procedure to yield the same results for a series of replicate determinations, included method and system reproducibility. System reproducibility was determined by performing GC analyses on 10 replicate 50 μL injections of the headspace from a single digested standard addition reference. Results showed a system reproducibility of 3.21% RSD.

Method reproducibility was determined for both Enfamil and ProSobee by analysis of 10 replicate 10 mL aliquots from a single can of each product. Individual results for Enfamil ranged from 113.6 to 146.8 mg choline/qt, with a standard deviation (SD) of 12.17, and RSD of 9.59%. Individual results for ProSobee ranged from 145.8 to 184.7 mg/qt (SD, 12.97; RSD, 7.70%).

Recovery studies were performed to determine the proper digestion time for maximum TMA production and thus for choline recovery. Spiked Enfamil and Prosobee samples were analyzed along with appropriate choline–water standards after

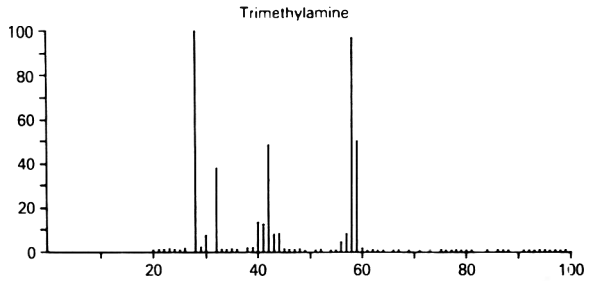

Figure 5. Mass spectrum of trimethylamine in Enfamil.

digestion times of 16, 22–24, and 48 h. Data indicate that 22–24 h is the optimum digestion time for maximum recovery.

A separate study was conducted to examine the possibility of a positive bias resulting from the presence of other quaternary-N compounds in liquid infant formula. Based on nonfat milk and demineralized whey formulations, carnitine (α-aminobutyric-β-hydroxy acid) may be present at levels up to 80 nM/mL in Enfamil (7). The study compared a GC-headspace analysis of Enfamil spiked with the equivalent of 80 nM carnitine/mL in unspiked Enfamil. Average choline levels were 89 and 86 mg/mL in spiked and unspiked samples, respectively. These levels are essentially the same when expected method error is considered, therefore indicating no contribution from carnitine.

Applicability of the system was shown by analyzing 9–10 different batches of each infant formula. All results conform to product expectations: For 9 Enfamil samples, results were 134.8, 146.8, 169.8, 153.2, 172.9, 164.1, 155.6, 155.9, and 118.1 mg choline/qt. Results on the 10 ProSobee samples analyzed were 164.9, 166.4, 186.7, 198.3, 191.5, 175.2, 176.4, 185.2, 185.6, and 170.1 mg choline/qt.

The simplicity of the manual method and ease of sample preparation allow a throughput of 160 samples/week, requiring only 35 work-hours.

Automated Headspace Analysis

The method was automated by using a headspace autosampler. The manual procedure was modified as described in *Method* to increase the speed and reliability of the automated analysis and to extend the use of the method to other formula products, including powders.

Automated system precision was evaluated by preparing a standard aqueous solution of TMA and ethyl ether at approximately equal concentrations. Ten vials of the standard were prepared, allowed to equilibrate at 40°C in the autosampler, and analyzed on the headspace analyzer. The relative standard deviation of the peak response factors was 1.80%.

The linearity of the FID response was evaluated by analyzing 8 sample types: Enfamil (whey-casein based infant formula in 20 Kcal/oz and 40 Kcal/oz concentrations), ProSobee (soy-based infant formula also in ready-to-use and concentrated preparations), Isocal (complete soy-based liquid diet), Sustacal (flavored liquid diet), Enfalac Powder (whey-casein based infant formula in powdered form), and ProSobee Powder (soy-based powdered infant formula). In each case, a series of samples was prepared as outlined in the method and spiked with increasing levels of choline chloride. The final concentrations of choline in the samples ranged from 0.033 to 0.167 mg/mL for each sample type tested. Only one vial was prepared at each concentration level because this most closely represented the ideal case for routine testing. Excellent linearity was demonstrated for each sample type (Table 1), with correlation coefficients ranging from 0.990 to 0.999

Table 1. Linearity and precision data for determination of choline by GC-headspace analysis

Sample	Correlation coefficient (5 points—individual vials)	%RSD	n
Enfami 20 Kcal/oz	0.997	6.91	10
Enfami 40 Kcal/oz	0.990	5.36	10
Isocal liquid	0.990	4.07	10
Sustacal liquid	0.998	1.60	8
ProSobee 20 Kcal/oz	0.997	4.74	6
ProSobee 40 Kcal/oz	0.993	5.28	6
ProSobee powder	0.999	3.73	6
Enfalac powder	0.999	5.64	7

Table 2. Comparison of time required and throughput for quantitative determination of choline by various methods

Time and No. samples analyzed	Reineckate procedure	GC-headspace	
		Manual	Automated
Time required:			
For analysis of 60 samples	5 days	3 days	2 days
Analyst time for 60 samples	40 h	20 h	6 h
Sample throughput:			
Per day	15	40	100
Per week	60	160	400

when response factors were plotted vs choline concentration. The slopes of the correlation lines generally ranged from 100 to 200 units per 0.1 mg/mL increase in choline concentration.

For each product type tested, a blank containing only product material digested at 120°C for 24 h was run on the headspace analyzer system. In addition, a reagent blank containing only digested 50% NaOH was prepared and analyzed. No interferences were found that contributed significantly to the TMA or ethyl ether peak areas.

Sample precision was determined for each sample type by preparing replicate vials as outlined in the method and analyzing them on the analyzer system. RSD values ranging from 1.60 to 6.91% were obtained (Table 1). This precision is excellent compared with current methodology, and based on the acceptance criteria for choline in all of the nutritional products tested (8).

Table 2 shows a comparison of the time required to perform the choline procedure by various methods. Automated headspace analysis requires only 15% of the time required by the Reineckate method. Sixty samples can be completed in 2 days with only 6 h analyst hands-on time. A throughput of up to 400 samples/week is possible, using only one full-time analyst. In a quality control laboratory, these statistics represent a large saving in analysis costs with a very short payback period for the cost of instrumentation.

Conclusions

Gas chromatographic headspace analysis is a rapid and reliable means of quantitating choline in nutritional liquids and powders. The procedure can be easily automated with commercially available instrumentation at reasonable cost. Automation significantly improved method precision. Only a few modifications in sample preparation rendered the method applicable to a variety of liquid and powder formula products. Headspace analysis is a more reproducible technique than is classical methodology, and is particularly suited to large volume quality control applications.

Acknowledgments

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Inductively Coupled Plasma Emission Spectroscopic Determination of Nine Elements in Infant Formula: Collaborative Study

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Results from a collaborative study of a method for the determination of 9 elements in infant formula, using inductively coupled plasma emission spectroscopy, are reported. Six collaborators analyzed 6 infant formulas for native and spiked levels of Ca, Cu, Fe, Mg, Mn, P, K, Na, and Zn. The within-laboratory and between-laboratory coefficients of variation were generally (69 of 108 samples) below 9% for all elements determined in all samples. Most of the average recoveries of the elements from spiked samples ranged from 90 to 105%. The method has been adopted official first action for determining Ca, Cu, Fe, Mg, Mn, P, K, Na, and Zn in infant formula.

In the last half of 1979, pediatricians reported an abnormally high incidence of hypochloremic metabolic alkalosis in infants. Many of these cases, characterized by a failure of the infant to thrive, were found to have resulted from an insufficient intake of chloride (1–5). The deficiency of chloride in the diet was traced to the almost exclusive use of 2 soy-based infant formulas that were low in chloride. Because the entire food intake of an infant may be limited to one formula, they are especially subject to the effects of a deficiency or excess of any component in the formula.

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The recommendation of the Associate Referee was approved by the General Referee and Committee E and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1984) **67**, March issue.

This report of the Associate Referee, R. F. Suddendorf, was presented at the 97th Annual International Meeting of the AOAC, Oct. 3–6, 1983, at Washington, DC.

Received September 7, 1983.

Table 1. Summary of instrumentation used in collaborative study

Instrument	Spectrometer	Nebulizer/ uptake rate, mL/min	Observation ht, mm, above load coil	Forward power, kW	Emission lines, nm, used other than those in Table 43:09
Applied Research Labs (34000)	direct reader	concentric/2.7	14	1.0	Mg, 279.1
Applied Research Labs (ICPQ)	direct reader	concentric/2.6	17	1.6	Ca, 422.7; Mg, 279.1; K, 404.7; Na, 330.2; Zn, 202.5; P, 213.6
Jarrell-Ash 975	direct reader	adjustable crossflow/0.9[a]	16	1.1	Ca, 370.6; Mg, 279.5; Zn, 206.2
Jarrell-Ash 975	direct reader	adjustable crossflow/1.1[a]	16	1.1	
Jarrell-Ash 1140	direct reader	nonadjustable crossflow/2.1[a]	15	0.9	Na, 330.2
Perkin-Elmer 5000	scanning	concentric/1.3[a]	11	1.25	Mg, 279.5

[a]Pump used to supply sample to nebulizer.

Table 43:09 Suggested operating parameters for ICP emission spectroscopy

Element	Wavelength, nm	Background correction[a]	Stds, μg/mL Low	Stds, μg/mL High
Ca	317.9	N	0	200
Cu	324.7	H	0	5
Fe	259.9	N	0	10
K	766.5	N	0	200
Mg	383.2	N	0	5
Mn	257.6	N	0	5
Na	589.0	N	0	200
P	214.9	N	0	100
Zn	213.8	H	0	5

[a]H = high side; N = no correction.

To prevent the occurrence of similar incidences, the U.S. government established a nutrient content standard for infant formulas and manufacturing requirements to ensure the safety of these products. In addition, the Food and Drug Administration (FDA) initiated a program to collect and analyze samples of infant formula for nutrients including 10 elements (Ca, Cu, Fe, Mg, Mn, P, K, Na, Zn, and Cl). To conduct this program, a rapid and accurate method of analysis was required. This manuscript provides data from a collaborative study supporting the suitability of inductively coupled plasma emission spectroscopy for determining 9 of these elements (all except Cl).

Collaborative Study

The study consisted of the analysis of 6 infant formulas (2 powders, 2 ready-to-feed, and 2 concentrates) for 9 elements: Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn. Each infant formula was analyzed in duplicate at the native (unspiked) level and again in duplicate at a spiked level. Two reagent blanks were analyzed by each collaborator. All collaborators analyzed the samples blind without any knowledge of sample composition.

All samples were preweighed and spiked in individual containers before being sent to collaborating laboratories. Collaborators were instructed to transfer the entire contents of each sample container to a Kjeldahl flask and to wash the container twice with ca 3 mL distilled deionized water and add the washings to the flask. Calculations were performed to reflect the elemental concentration as μg/mL or mg/mL for the ready-to-feed and concentrated infant formulas and as μg/g or mg/g for the powdered formulas with blank values subtracted. Thus, all concentrations are for the product as taken directly from the can or container and do not reflect normal dilutions of the product prior to feeding.

Calcium, Copper, Iron, Magnesium, Manganese, Phosphorus, Potassium, Sodium, and Zinc in Infant Formula

Inductively Coupled Plasma Emission Spectroscopy Method

First Action

(*Caution: See* **51.019, 51.026, 51.028.**)

Principle

Sample is digested in $HNO_3/HClO_4$ and elements are detd by ICP emission spectroscopy.

Reagents

(a) *Water.*—Distd, deionized. Use thruout.

(b) *Perchloric acid.*—Double-distd (G. F. Smith, or equiv.). Dil. 10 mL to 50 mL with H_2O.

(c) *Standards.*—(All preps yield 1000 μg/mL.)

(1) Calcium.—Place 2.4973 g $CaCO_3$ in 1 L vol. flask with 300 mL H_2O, add 10 mL HCl, after CO_2 has been released, dil. to 1 L with H_2O.

(2) Copper.—Dissolve 1.000 g Cu in 10 mL HCl plus 5 mL H_2O to which HNO_3 is added dropwise until dissolution is complete. Boil to expel fumes, cool, and dil. to 1 L with H_2O.

(3) Iron.—Dissolve 1.000 g Fe wire in 20 mL 5M HCl; dil. to 1 L with H_2O.

(4) Potassium.—Dissolve 1.9067 g KCl in H_2O and dil. to 1 L with H_2O.

(5) Magnesium.—Dissolve 1.000 g Mg in 50 mL 1M HCl and dil. to 1 L with H_2O.

(6) Manganese.—Dissolve 1.000 g Mn in 10 mL HCl plus 1 mL HNO_3 and dil. 1 L with H_2O.

(7) Sodium.—Dissolve 2.5421 g NaCl in H_2O and dil. to 1 L with H_2O.

(8) Phosphorus.—Dissolve 4.263 g $(NH_4)_2HPO_4$ in H_2O and dil. to 1 L with H_2O.

(9) Zinc.—Dissolve 1.000 g Zn in 10 mL HCl and dil. to 1 L with H_2O.

Com. available certified stds may be substituted for any of the above elements.

(d) *Calibration stds.*—Prep. to concn indicated in Table **43:09,** using stds above. All calibration stds should be prepd to contain 20% $HClO_4$ to approx. $HClO_4$ concn in dild digested samples.

Apparatus

(a) *ICP emission spectrometer.*—Model 975 Plasma Atom Comp (Jarrell-Ash Co., 590 Lincoln St., Waltham, MA 02154), or equiv., capable of simultaneous or sequential detn of Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn. Minipuls 2 peristaltic pump (Gilson Medical Electronics, PO Box 27, 3000 W Beltline Highway, Middleton, WI 53562), or equiv., used to feed glass crossflow nebulizer. Suggested operating parameters: warmup time (plasma on), 30 min; exposure time, 5 s; integration cycles, 1 cycle (2 on line exposures, 1 off line exposure); forward power, 1.1 KW; reflected power, <5 watts; sample uptake rate (w/pump), 0.8 mL/min; observation ht: 16 mm above load coil (optimized for Mn).

(b) *Controllable heating mantle and acid scrubber.*—Labconco 60301, or equiv. (Labconco, 8811 Prospect, Kansas City, MO 64132).

Table 2. Collaborative results for Ca in infant formulas

Product[a]	Laboratory 1	2	3	4	5	6	Ref.	Spike	$\bar{x}$	Rec., %	S_o	S_L	S_x	CV_o	CV_L	CV_x
Brand A soy conc.																
Native, mg/mL	1.86	1.82	1.73	2.09	2.05	1.973	1.81	0								
	1.86	1.84	1.72	1.888		1.916	1.79	0	1.873	—	0.061	0.097	0.115	3.3	5.2	6.1
Spiked, mg/mL	3.74	3.79	3.61	4.309	4.35	5.669[b]		2.00								
	3.84	3.82	3.74	3.931	4.37	5.688[b]		2.00	3.950	104	0.131	0.267	0.297	3.3	6.8	7.5
Brand B RTF																
Native, mg/mL	0.49	0.47	0.442	0.5024	0.539	0.484	0.48	0								
		0.464	0.447	0.3783	0.526	0.486	0.49	0	0.477	—	0.036	0.018	0.041	7.6	3.8	8.5
Spiked, mg/mL	1.17	1.12	1.04	1.339		1.17		0.67								
	1.12	1.13	1.05	1.252		1.15		0.67	1.154	101	0.033	0.088	0.094	2.8	7.6	8.1
Brand C RTF																
Native, mg/mL	0.63	0.62	0.633	0.627	0.74	0.689	0.77	0								
	0.70	0.64	0.626	0.6834	0.70	0.686	0.78	0	0.680	—	0.027	0.049	0.056	4.0	7.2	8.2
Spiked, mg/mL	1.32	1.27	0.933	1.278	1.56	1.326		0.67								
	1.27	1.31	1.17	1.033	1.57	1.256		0.67	1.275	89	0.102	0.157	0.187	8.0	12.3	14.7
Brand B powder																
Native, mg/g	3.32	3.4	3.27[b]	3.84		3.622	3.60	0								
	3.66	3.53	2.17[b]	3.792		3.667	3.61	0	3.604	—	0.117	0.113	0.163	3.3	3.2	4.5
Spiked, mg/g	6.88	6.75	6.18	6.529	7.69	6.717		3.33								
	6.57	6.68	5.97	6.409	7.69	6.954		3.33	6.752	95	0.134	0.526	0.543	2.0	7.8	8.0
Brand D powder																
Native, mg/g	4.08	4.01	3.78	3.723	4.45	4.008	4.05	0								
	4.17	4.07	3.74	3.833	4.44	4.013	4.1	0	4.033	—	0.045	0.229	0.233	1.1	5.7	5.8
Spiked, mg/g	10.2	10.7	9.79	11.837	12.2	10.948		6.67								
	10.5	10.5	10.00	12.637		11.105		6.67	10.947	104	0.290	0.918	0.962	2.7	8.4	8.8
Brand D conc.																
Native, mg/mL	1.18	1.12	1.14	1.232	1.29	1.156	1.12	0								
	1.15	1.15		1.246	1.25	1.131	1.11	0	1.175	—	0.019	0.058	0.061	1.6	5.0	5.2
Spiked, mg/mL	2.09	2.07	2.04	2.003	2.36	2.214		1.00								
	2.21	2.09	1.92	1.776	2.40	2.185		1.00	2.113	94	0.083	0.162	0.182	3.9	7.7	8.6

[a]Conc. = concentrate, RTF = ready to feed.
[b]Outlier, not used in statistical analysis.

Table 3. Collaborative results for Cu in infant formulas

Product[a]	Laboratory 1	2	3	4	5	6	Ref.	Spike	$\bar{x}$	Rec., %	S_o	S_L	S_x	CV_o	CV_L	CV_x
Brand A soy conc.																
Native, mg/mL	1.40	1.3	1.18	1.80[b]	1.36	1.51	1.24	0								
	1.30	1.27	1.16	2.40[b]		1.42	1.25	0	1.308	—	0.044	0.101	0.110	3.4	7.7	8.4
Spiked, mg/mL	5.67	5.8	5.49	6.55	6.22	7.51		5.00								
	5.83	5.9	5.50	6.40	6.37	6.81		5.00	6.171	97	0.218	0.584	0.623	3.5	9.5	10.1
Brand B RTF																
Native, mg/mL	0.37	0.46	0.45	0.73[b]	0.486	0.512	0.49	0								
		0.44	0.45	0.50[b]	0.465	0.526	0.51	0	0.469	—	0.012	0.044	0.046	2.6	9.4	9.7
Spiked, mg/mL	1.60	1.51	1.47	1.80		1.68		1.0								
	1.70	1.44	1.52	2.00		1.61		1.0	1.633	116	0.079	0.161	0.179	4.8	9.8	11.0
Brand C RTF																
Native, mg/mL	0.90	0.77	0.92	0.83	0.771	0.832	0.78	0								
	0.90	0.76	0.72	0.97	0.770	0.829	0.80	0	0.825	—	0.066	0.032	0.073	7.9	3.9	8.9
Spiked, mg/mL	2.00	2.15	1.26	1.90	2.00	2.43		1.33								
	2.30	1.92	1.77	1.57	2.11	1.75		1.33	1.930	83	0.287	0.145	0.321	14.9	7.5	16.7
Brand B powder																
Native, mg/g	5.04	3.6	3.60	6.67		4.09	4.62	0								
	4.29	3.8	2.30	6.67		4.14	4.70	0	4.460	—	0.438	1.222	1.298	9.8	27.4	29.1
Spiked, mg/g	12.8	14.6	13.3	16.33	14.4	14.9		11.3								
	12.7	14.6	13.4	16.67	14.3	16.7		11.3	14.558	89	0.531	1.370	1.469	3.7	9.4	10.1
Brand D powder																
Native, mg/g	6.31[b]	4.4	4.10	5.33	4.48	4.96	4.48	0								
	3.97[b]	4.4	4.50	6.00	4.57	4.93	4.35	0	4.708	—	0.230	0.494	0.545	4.9	10.5	11.6
Spiked, mg/g	3.1	15.4	14.4	17.00	14.9	17.5		11.3								
	4.2	14.9	14.2	18.00		17.4		11.3	15.546	96	0.501	1.647	1.721	3.2	10.6	11.1
Brand D conc.																
Native, mg/mL	1.05	1.15	1.15	1.80[b]	1.22	1.31	1.15	0								
	1.35	1.16		2.00[b]	1.19	1.32	1.09	0	1.195	—	0.097	0.0	0.097	8.2	0.0	8.2
Spiked, mg/mL	2.50	2.9	2.84	3.55	3.05	3.31		2.00								
	3.55	2.76	2.51	3.05	3.20	3.46		2.00	3.057	93	0.357	0.102	0.371	11.7	3.3	12.1

[a]Conc. = concentrate; RTF = ready to feed.
[b]Outlier, not used in statistical analysis.

(c) *Glassware.*—Soak overnight in 10% HNO_3 and thoroly rinse with H_2O.

Procedure

Vigorously shake container of infant formula to ensure complete mixing. Measure 15.0 mL of ready-to-feed (10.0 mL if concd, 1.5 g if powd) infant formula into 100 mL Kjeldahl flask. Add 30 mL HNO_3–$HClO_4$ (2 + 1) to flask along with 3 or 4 glass boiling beads. Let samples sit overnight in acid. Carry 2 reagent blanks thru entire procedure along with samples.

Before starting digestion, have ice bath available for cooling Kjeldahl flasks. HNO_3 should also be readily available. To start digestion, place each Kjeldahl flask on heating mantle

Table 4. Collaborative results for Fe in infant formulas

Product[a]	Laboratory 1	2	3	4	5	6	Ref.	Spike	$\bar{x}$	Rec., %	S_o	S_L	S_x	CV_o	CV_L	CV_x
Brand A soy conc.																
Native, mg/mL	29.3	28.3	24.6[b]	31.50	33.8	33.8	29.3	0								
	31.5	28.6	24.2[b]	31.80		32.4	29.1	0	30.85	—	0.84	1.93	2.11	2.7	6.3	6.8
Spiked, mg/mL	76.7	70.1	61.6[b]	84.50	84.7	87.1		50.0								
	79.0	70	62.6[b]	84.75	85.2	81.2		50.0	80.33	99	2.01	6.29	6.60	2.5	7.8	8.2
Brand B RTF																
Native, mg/mL	13.2	12.1	10.4[b]	13.83[b]	14.3	13.6	13.1	0								
		12.0	10.6[b]	11.20[b]	13.9	13.8	12.7	0	13.19	—	0.22	0.82	0.85	1.6	6.2	6.4
Spiked, mg/mL	26.9	23.5	20.2[b]	22.67		26.1		13.3								
	25.3	23.5	20.2[b]	22.67		26.6		13.3	24.66	86	0.59	1.80	1.89	2.4	7.3	7.7
Brand C RTF																
Native, mg/mL	16.8	16.8	14.3[b]	16.23	19.2	18.2	18	0								
	16.7	16.4	14.6[b]	16.83	18.8	18.3	18.5	0	17.12	—	0.27	1.51	1.54	1.6	8.8	9.0
Spiked, mg/mL	49.6	44.6	30.6[b]	43.67	52.8	49.6		33.3								
	46.6	44.5	37.5[b]	32.90	52.6	48.8		33.3	44.48	82	3.80	6.54	7.57	8.5	14.7	17.0
Brand B powder																
Native, mg/g	90.1[b]	94	86.2[b]	109.0		108	102	0								
	105[b]	99	56.0[b]	106.7		110	102	0	103.84	—	2.07	5.58	5.96	2.0	5.4	5.7
Spiked, mg/g	227	212	183[b]	199.7	246	238		133								
	218	209	180[b]	221.3	244	236		133	217.83	86	6.91	22.07	23.13	3.2	10.1	10.6
Brand D powder																
Native, mg/g	3.15	2.3	1.20[b]	7.33	2.93	2.63	2.5	0								
	3.31	2.1	1.30[b]	7.00	8.38	11.3	2.6	0	4.63	—	2.96	0.80	3.07	63.9	17.4	66.3
Spiked, mg/g	12.1	13.4	10.0[b]	16.33	16.3	14.2		13.3								
	16.8	13.1	10.0[b]	19.00		14.9		13.3	15.13	79	1.93	1.06	2.20	12.8	7.0	14.6
Brand D conc.																
Native, mg/mL	4.85	4.29	3.72[b]	4.75	5.22	4.95	4.45	0								
	4.40	4.4		4.45	5.13	4.94	4.39	0	4.69	—	0.16	0.30	0.34	3.5	6.3	7.2
Spiked, mg/mL	9.30	8.5	7.25[b]	8.15	10.1	9.97		5.0								
	9.70	8.5	6.91[b]	7.25	10.17	9.78		5.0	9.14	89	0.32	0.99	1.04	3.5	10.9	11.4

[a]Conc. = concentrate, RTF = ready to feed.
[b]Outlier, not used in stat stical analysis.

Table 5. Collaborative results for K in infant formulas

Product[a]	Laboratory 1	2	3	4	5	6	Ref.	Spike	$\bar{x}$	Rec., %	S_o	S_L	S_x	CV_o	CV_L	CV_x
Brand A soy conc.																
Native, mg/mL	1.81	1.74		1.972	1.98	2.018	1.88	0								
	1.83	1.8		1.799		1.943	1.68	0	1.859	—	0.089	0.064	0.110	4.8	3.4	5.9
Spiked, mg/mL	2.73	2.7	3.22[b]	3.164	3.07	3.344		1.00								
	2.82	2.7	2.40[b]	3.052	3.08	3.046		1.00	2.971	111	0.105	0.206	0.231	3.5	6.9	7.8
Brand B RTF																
Native, mg/mL	0.69	0.68		0.7213[b]	0.711	0.726	0.64	0								
	0.69	0.69		0.5676[b]	0.726	0.745	0.69	0	0.699	—	0.018	0.025	0.031	2.6	3.6	4.4
Spiked, mg/mL	1.40	1.35	1.24	1.455		1.475		0.67								
	1.35	1.31	1.27	1.352		1.422		0.67	1.362	99	0.043	0.067	0.080	3.2	5.0	5.9
Brand C RTF																
Native, mg/mL	0.78	0.78		0.7366	0.792	0.814	0.75	0								
	0.77	0.76		0.8473	0.781	0.792	0.85	0	0.788	—	0.044	0.0	0.044	5.6	0.0	5.6
Spiked, mg/mL	1.43	1.39	1.20	1.291	1.40	1.50		0.67								
	1.47	1.41		1.129	1.41	1.475		0.67	1.373	87	0.054	0.111	0.123	3.9	8.0	8.9
Brand B powder																
Native, mg/g	4.33	4.4		5.259		4.951	4.67	0								
	4.82	4.7		5.127		5.019	4.66	0	4.794	—	0.188	0.250	0.312	3.9	5.2	6.5
Spiked, mg/g	10.9	10.8		13.107	11.10	11.661		6.67								
	10.5	11.0		13.140	11.00	11.796		6.67	11.500	101	0.151	0.980	0.992	1.3	8.5	8.6
Brand D powder																
Native, mg/g	9.26	9.0		8.66[b]	9.59	9.989	8.9	0								
	9.37	9.2		10.79[b]	9.72	9.794	9.2	0	9.402	—	0.140	0.349	0.376	1.5	3.7	4.0
Spiked, mg/g	14.7	16	12.5	17.38	15.40	16.806		6.67								
	15.2	15.1	12.4	18.24		17.067		6.67	15.527	92	0.433	1.907	1.956	2.8	12.3	12.6
Brand D conc.																
Native, mg/mL	1.58	1.6		1.798	1.81	1.794	1.65	0								
	1.57	1.65		1.871	1.75	1.799	1.63	0	1.725	—	0.032	0.086	0.092	1.8	5.0	5.3
Spiked, mg/mL	2.54	2.5	2.38	2.582	2.87	2.816		1.0								
	2.70	2.5	2.57	2.144	2.77	2.522		1.0	2.575	85	0.171	0.108	0.202	6.6	4.2	7.9

[a]Conc. = concentrate, RTF = ready to feed.
[b]Outlier, not used in statistical analysis.

set at low temp. Once boiling is initiated, red-orange fumes of NO_2 will be driven off. Continue gentle heating until HNO_3 and H_2O have been driven off, and sample is contained in $HClO_4$. At this point effervescent reaction occurs between sample and $HClO_4$. Place flask on cool heating mantle and let digestion proceed with occasional heating from mantle. It is important that reaction between sample and $HClO_4$ not go too fast, because charring will occur. If sample chars, immediately place flask in ice bath to stop digestion. Add 1 mL HNO_3 and resume gentle heating.

After reaction of sample with $HClO_4$ is complete (identified by cessation of effervescent reaction between sample and

Table 6. Collaborative results for Mg in infant formulas

Product[a]	Laboratory 1	2	3	4	5	6	Ref.	Spike	$\bar{x}$	Rec., %	S_o	S_L	S_x	CV_o	CV_L	CV_x
Brand A soy conc.																
Native, mg/mL	200	213	198	269	239	221	200	0								
	201	213	193	253.5		212	199	0	216.27	—	5.39	23.91	24.51	2.5	11.1	11.3
Spiked, mg/mL	415	384	362	454.0	445	440		200								
	429	387	350	457.0	454	403		200	415.00	99	12.27	37.14	39.11	3.0	9.0	9.4
Brand B RTF																
Native, mg/mL	70.6	77	71.1	83.3[b]	80.9	73.7	70.4	0								
		76	72.6	57.2[b]	78.7	75.2	72.7	0	74.45	—	1.25	3.37	3.59	1.7	4.5	4.8
Sp ked, mg/mL	131	138	126	173.3[b]		137		66.7								
	126	138	127	159.7[b]		135		66.7	132.25	87	1.94	5.44	5.77	1.5	4.1	4.4
Brand C RTF																
Native, mg/mL	57.6	62	55.6	56.0[b]	61.8	57.5	57.5	0								
	56.9	60	55.7	64.2[b]	60.9	57.7	58.8	0	58.50	—	0.77	2.17	2.30	1.3	3.7	3.9
Spiked, mg/mL	113	120	82.0[b]	130.3	125	120		66.7								
	116	121	102[b]	108.0	126	115		66.7	119.43	91	7.30	0.0	7.30	6.1	0.0	6.1
Brand B powder																
Native, mg/g	397	440	410[b]	503.3		427	422	0								
	429	454	280[b]	503.3		431	418	0	442.46	—	11.19	35.44	37.17	2.5	8.0	8.4
Spiked, mg/g	980	1080	988	1236.7	1090	1016		667								
	941	1070	954	1260.0	1090	1045		667	1062.56	93	18.62	104.29	105.94	1.8	9.8	10.0
Brand D powder																
Native, mg/g	408	431	389	408.3[b]	444	416	393	0								
	410	437	394	475.0[b]	447	412	396	0	414.75	—	2.87	21.35	21.55	0.7	5.2	5.2
Spiked, mg/g	652	760	669	816.7	752	730		333								
	670	740	680	835.0		742		333	731.52	95	11.51	61.80	62.87	1.6	8.5	8.6
Brand D conc.																
Native, mg/mL	114	118	116	130.2	131	119	117	0								
	112	121		130.5	127	119	116	0	120.82	—	1.58	6.67	6.86	1.3	5.5	5.7
Spiked, mg/mL	189	206	196	207.0[b]	226	213		100								
	198	208	186	176.0[b]	226	209		100	205.70	85	4.48	13.84	14.55	2.2	6.7	7.1

[a]Conc. = concentrate, RTF = ready to feed.
[b]Outlier, not used in statistical analysis.

Table 7. Collaborative results for Mn in infant formulas

Product[a]	Laboratory 1	2	3	4	5	6	Ref.	Spike	$\bar{x}$	Rec., %	S_o	S_L	S_x	CV_o	CV_L	CV_x
Brand A soy conc.																
Native, mg/mL	3.00	3.02	2.59[b]	2.95	3.23	3.43	2.89	0								
	3.00	3.03	2.58[b]	2.90		3.31	2.89	0	3.059	—	0.041	0.187	0.191	1.4	6.1	6.3
Spiked, mg/mL	7.26	7.59	6.69[b]	7.50	8.29	9.13		4.5								
	7.53	7.6	6.65[b]	7.05	8.35	8.53		4.5	7.681	103	0.231	0.758	0.792	3.0	9.9	10.3
Brand B RTF																
Native, mg/mL	0.13	0.223	0.07[b]	0.23	0.215	0.207	0.24	0								
		0.221	0.07[b]	0.17	0.205	0.219	0.22	0	0.207	—	0.021	0.025	0.032	9.9	11.9	15.5
Spiked, mg/mL	0.40[b]	0.501	0.30[b]	0.47		0.479		0.27								
	0.53[b]	0.50	0.33[b]	0.47		0.470		0.27	0.482	102	0.004	0.016	0.017	0.8	3.4	3.5
Brand C RTF																
Native, mg/mL	0.60[b]	0.525	0.34[b]	0.50	0.529	0.517	0.50	0								
	0.60[b]	0.51	0.35[b]	0.53	0.516	0.518	0.52	0	0.517	—	0.013	0.0	0.013	2.5	0.0	2.5
Soiked, mg/mL	1.30	1.71	1.09[b]	1.53	1.94	1.59		1.33								
	1.57	1.69	1.18[b]	1.33	1.66	1.75		1.33	1.607	82	0.147	0.130	0.196	9.2	8.1	12.2
Brand B powder																
Native, mg/g	2.82[b]	1.61		1.67		1.53	1.76	0								
	1.65[b]	1.71		1.67		1.54	1.70	0	1.649	—	0.041	0.076	0.087	2.5	4.6	5.3
Spiked, mg/g	3.22[b]	4.45	2.60[b]	4.67	4.28	4.07		2.67								
	3.22[b]	4.4	2.40[b]	4.33	4.3	4.21		2.67	4.339	101	0.131	0.128	0.184	3.0	3.0	4.2
Brand D powder																
Native, mg/g	2.03[b]	0.31		0.33[b]	0.321	0.287	0.34	0								
		0.34		0.66[b]	0.320	0.310	0.37	0	0.325	—	0.017	0.020	0.026	5.3	6.1	8.1
Spiked, mg/g	2.02	2.90	1.50[b]	3.33	3.08	2.97		2.67								
	2.11	2.8	1.50[b]	3.67		3.00		2.67	2.876	96	0.130	0.543	0.558	4.5	18.9	19.4
Brand D conc.																
Native, mg/mL	0.22[b]	0.124		0.15	0.120	0.110	0.11	0								
		0.124		0.15	0.110	0.109	0.11	0	0.122	—	0.003	0.017	0.017	2.6	13.7	14.0
Spiked, mg/mL	0.40	0.51	0.27[b]	0.45	0.509	0.527		0.40								
	0.60	0.51	0.25[b]	0.40	0.527	0.517		0.40	0.495	93	0.066	0.0	0.066	13.2	0.0	13.2

[a]Conc. = concentrate, RTF = ready to feed.
[b]Outlier, not used in statistical analysis.

$HClO_4$) apply high heat to sample for ca 2 min. Avoid heating sample to dryness because this can cause explosion. Remove flask from heating mantle and let cool.

Transfer each digested sample to 50 mL vol. flask and dil. to vol. with H_2O. Some pptn is likely to occur (especially with high salt content samples) after diln. Ppt will dissolve if shaken and allowed to sit overnight. Final acid content of samples is ca 20% $HClO_4$.

Elemental detn is accomplished by inductively coupled plasma (ICP) emission spectroscopy (see Table **43:09** for parameters). Calibration of instrument is done thru use of known calibration stds. Calibration stds may be used as single

Table 8. Collaborative results of Na in infant formulas

Product[a]	Laboratory 1	2	3	4	5	6	Ref.	Spike	$\bar{x}$	Rec., %	S_o	S_L	S_x	CV_o	CV_L	CV_x
Brand A soy conc.																
Native, mg/mL	0.68	0.72	0.691	0.6776	0.772	0.799	0.71	0								
	0.75	0.72	0.668	0.6534		0.774	0.66	0	0.713	—	0.028	0.040	0.049	3.9	5.7	6.9
Spiked, mg/mL	1.59	1.66	1.67	2.02	1.75	1.984		1.00								
	1.69	1.68	1.54	1.908	1.78	1.826		1.00	1.758	105	0.074	0.138	0.157	4.2	7.9	8.9
Brand B RTF																
Native, mg/mL	0.24[b]	0.158	0.135	0.1677	0.177	0.169	0.16	0								
		0.154	0.16	0.1319	0.158	0.17	0.17	0	0.159	—	0.014	0.0	0.014	8.9	0.0	8.9
Spiked, mg/mL	0.52	0.48	0.479	0.4309		0.514		0.33								
	0.49	0.48	0.488	0.4566		0.510		0.33	0.485	99	0.013	0.025	0.028	2.7	5.2	5.8
Brand C RTF																
Native, mg/mL	0.30	0.32	0.317	0.3119	0.306	0.335	0.33	0								
	0.30	0.32	0.314	0.3256	0.301	0.334	0.35	0	0.319	—	0.007	0.014	0.015	2.1	4.4	4.8
Spiked, mg/mL	0.68	0.62	0.486	0.5485	0.58	0.68		0.33								
	0.61	0.60	0.526	0.4845	0.587	0.65		0.33	0.588	82	0.032	0.062	0.069	5.4	10.5	11.8
Brand B powder																
Native, mg/g	1.01	1.08		1.351		1.195	1.14	0								
	1.14	1.13		1.311		1.20	1.13	0	1.169	—	0.046	0.096	0.107	3.9	8.2	9.1
Spiked, mg/g	2.38	2.42	2.22	2.331	2.41	2.338		1.33								
	2.29	2.3	2.15	2.356	2.23	2.449		1.33	2.323	87	0.078	0.046	0.091	3.4	2.0	3.9
Brand D powder																
Native, mg/g	2.18	2.29	2.05	2.257	2.34	2.475	2.20	0								
	2.18	2.3	2.16	2.491	2.408	2.477	2.27	0	2.291	—	0.074	0.117	0.138	3.2	5.1	6.0
Spiked, mg/g	5.17	5.4	5.47	4.967	4.98	5.902		3.33								
	5.25	5.3	5.50	4.676		5.94		3.33	5.323	91	0.102	0.387	0.400	1.9	7.3	7.5
Brand D conc.																
Native, mg/mL	0.50	0.52	0.55	0.4666[b]	0.562	0.577	0.52	0								
	0.50	0.538		0.5188[b]	0.549	0.578	0.51	0	0.537	—	0.008	0.029	0.030	1.4	5.4	5.6
Spiked, mg/mL	0.98	0.967	1.02	0.7860[b]	0.999	1.103		0.50								
	1.04	0.99	0.965	0.7626[b]	1.01	1.087		0.50	1.016	96	0.027	0.041	0.050	2.7	4.1	4.9

[a]Conc. = concentrate, RTF = ready to feed.
[b]Outlier, not used in statistical analysis.

Table 9. Collaborative results for P in infant formulas

Product[a]	Laboratory 1	2	3	4	5	6	Ref.	Spike	$\bar{x}$	Rec., %	S_o	S_L	S_x	CV_o	CV_L	CV_x
Brand A soy conc.																
Native, mg/mL	1.37	1.30	1.20	1.559	1.49	1.523	1.29	0								
	1.36	1.31	1.19	1.352		1.526	1.28	0	1.365	—	0.060	0.112	0.127	4.4	8.2	9.3
Spiked, mg/mL	2.39	2.27	2.10	2.46	2.66	3.146		1.00								
	2.45	2.27	2.04	2.399	2.69	2.65		1.00	2.460	110	0.147	0.274	0.311	6.0	11.1	12.6
Brand B RTF																
Native, mg/mL	0.34	0.332	0.297	0.3426	0.374	0.346	0.33	0								
		0.329	0.303	0.2591	0.366	0.357	0.34	0	0.332	—	0.025	0.019	0.031	7.4	5.8	9.4
Spiked, mg/mL	0.70	0.66	0.586	0.771		0.697		0.33								
	0.68	0.66	0.595	0.7052		0.70		0.33	0.675	104	0.022	0.053	0.057	3.3	7.8	8.5
Brand C RTF																
Native, mg/mL	0.40	0.391	0.386	0.3937	0.458	0.453	0.47	0								
	0.45	0.40	0.393	0.4472	0.43	0.464	0.48	0	0.430	—	0.022	0.028	0.035	5.0	6.4	8.2
Spiked, mg/mL	1.09	1.03	0.71	1.05	1.21	1.119		0.67								
	1.05	1.04	0.889	0.8543	1.22	1.159		0.67	1.035	90	0.078	0.135	0.156	7.6	13.1	15.1
Brand B powder																
Native, mg/g	2.40	2.46	2.26[b]	2.565		2.688	2.51	0								
	2.63	2.56	1.50[b]	2.807		2.68	2.54	0	2.584	—	0.111	0.049	0.121	4.3	1.9	4.7
Spiked, mg/g	5.89	5.70	5.12	6.393	6.26	5.884		3.33								
	5.60	5.6	4.96	6.467	6.23	6.079		3.33	5.849	98	0.117	0.486	.500	2.0	8.3	8.6
Brand D powder																
Native, mg/g	3.28	3.21	2.88	2.974	3.52	3.428	3.14	0								
	3.34	3.26	2.89	3.59	3.54	3.332	3.2	0	3.256	—	0.169	0.160	0.233	5.2	4.9	7.2
Spiked, mg/g	6.32	6.5	5.78	7.91	7.18	6.898		3.33								
	6.57	6.4	5.82	7.64		6.876		3.33	6.718	104	0.121	0.696	0.707	1.8	10.4	10.5
Brand D conc.																
Native, mg/mL	0.95	0.89	0.866	0.961	1.02	0.98	0.86	0								
	0.93	0.91		0.9605	0.994	0.974	0.86	0	0.935	—	0.011	0.055	0.056	1.2	5.8	6.0
Spiked, mg/mL	1.90	1.82	1.74	1.777	2.07	2.072		1.0								
	1.99	1.84	1.64	1.568	2.13	2.232		1.0	1.898	96	0.087	0.194	0.212	4.6	10.2	11.2

[a]Conc. = concentrate, RTF = ready to feed.
[b]Outlier, not used in statistical analysis.

stds (i.e., one element per std) or as mixed std contg 2 or more elements. Whether single or mixed stds should be used for calibration will depend on computer software requirements of particular ICP system in use.

After calibration is complete, samples can be analyzed. Calibration of instrument should be checked after every 10 samples by analyzing calibration stds. If reanalysis of calibration stds indicates that instrument has drifted out of calibration, (>3% of original values), instrument should be recalibrated.

Computer will calc. concn for each element of each dild sample as μg/mL. Use following equation to convert this

Table 10. Collaborative results for Zn in infant formulas

Product[a]	Laboratory 1	2	3	4	5	6	Ref.	Spike	$\bar{x}$	Rec., %	S_o	S_L	S_x	CV_o	CV_L	CV_x
Brand A soy conc.																
Native, mg/mL	11.3	11.0	11.1	13.05[b]	13.0	13.4	11.8	0								
	11.8	11.2	11.1	59.75[b]		13.1	11.7	0	11.86	—	0.20	0.91	0.93	1.7	7.7	7.8
Spiked, mg/mL	19.3	19.2	19.1	18.70	23.9	24.6		10								
	20.0	19.3	18.4	20.10	24.2	23.7		10	20.88	90	0.57	2.48	2.55	2.7	11.9	12.2
Brand B RTF																
Native, mg/mL	2.46	3.6	3.63	4.67	4.29	4.15	3.81	0								
		3.56	3.71	3.40	4.14	4.14	3.87	0	3.80	—	0.370	0.403	0.547	9.7	10.6	14.4
Spiked, mg/mL	9.22	9.5	9.38	10.80		11.2		6.67								
	9.12	9.5	9.45	10.37		10.9		6.67	9.94	92	0.170	0.814	0.832	1.7	8.2	8.4
Brand C RTF																
Native, mg/mL	5.50	5.8	5.66	6.07	6.59	6.30	6.11	0								
	5.56	5.6	5.65	6.40	6.50	6.19	6.34	0	6.02	—	0.127	0.375	0.396	2.1	6.2	6.6
Spiked, mg/mL	11.4	11.4	8.40	12.27	14.1	13.6		6.67								
	11.9	11.3	10.6	10.13	13.9	12.8		6.67	11.82	87	0.93	1.45	1.72	7.9	12.3	14.6
Brand B powder																
Native, mg/g	22.2	26.5	27.2	88.67[b]		31.2	31.1	0								
	28.7	27.5	18.2	31.00[b]		31.6	29.6	0	27.38	—	3.56	2.52	4.36	13.0	9.2	15.9
Spiked, mg/g	79.6	87	87.3	101.0	110	96.4		66.7								
	75.0	86	83.1	102.7	108	98.5		66.7	92.88	98	2.06	11.77	11.95	2.2	12.7	12.9
Brand D powder																
Native, mg/g	47.8	48	48.0	48.00	53.7[b]	55.0	50.3	0								
	45.3	49	48.4	54.33	65.9[b]	54.7	51.1	0	49.99	—	2.00	2.56	3.25	4.0	5.1	6.5
Spiked, mg/g	95.8	108	105	111.0	135	123		66.7								
	99.7	106	107	114.0		125		66.7	111.77	93	1.90	12.08	12.23	1.7	10.8	10.9
Brand D conc.																
Native, mg/mL	11.1	10.9	11.6	12.50	13.0	13.2	11.4	0								
	11.1	11.2		13.00	12.5	13.0	11.2	0	11.98	—	0.24	0.90	0.93	2.0	7.5	7.7
Spiked, mg/mL	19.1	19.2	19.9	19.55	22.9	23.6		10								
	20.7	19.4	18.9	17.00	23.6	23.2		10	20.59	86	0.95	2.08	2.29	4.6	10.1	11.1

[a]Conc. = concentrate, RTF = ready to feed.
[b]Outlier, not used in statistical analysis.

value to μg/mL if original sample was ready-to-feed or concd formula or μg/g if sample was powder.

$$C = A \times (50 \text{ mL}/B)$$

where A = concn (μg/mL) of element as detd by ICP; B = vol. or wt of sample as mL or g; C = elemental concn in sample, μg/mL or μg/g, depending on value of B.

Results and Discussion

Six laboratories completed the study; a summary of the instrumentation used by each laboratory is given in Table 1. Results of the analysis are shown in Tables 2–10. The statistical parameters calculated after removal of outliers as determined by the Youden rank sum test (6), the Dixon test (6), and the Cochran test (6) are also shown. All data and statistics are reported as received from the laboratories and statistician: no attempt has been made to round numbers to the correct number of significant figures.

In applying these outlier tests, first the Youden rank sum test was applied at $\alpha = 0.01$ to all data. All Fe and Mn values from Laboratory 3 were rejected as a result of this test. Next, the Dixon and Cochran tests were applied concurrently to the remaining data at $\alpha = 0.05$ for both tests. The Dixon test examines the high and the low end of the data with $\alpha = 0.05$ at each tail. To avoid removal of noninfluential outliers in these tests, Dixon outliers were not removed if such removal did not change CV_x by at least 40% of its original value (in the general case, the 40% restriction may be replaced by a confidence interval), and Cochran outliers were not removed if the ratio of CV_o to CV_x fell below 0.25, a value established empirically. These 2 limitations prevented removal of an excessive number of data points for a material when the remaining points were unusually close together. In addition, as a general rule, no more than 20% of the data for a given material were removed as outliers, a value permitting removal of one laboratory from a 5-laboratory set, but no more. One exception to this rule is found in Table 7 for Mn. For the Brand D powder formula, 2 laboratories were removed (a removal rate of 33%) because both were extreme outliers and most likely were not true members of the normal distribution of data. Other than this exception, all data were treated in the same manner as described by the above rules.

In the statistical treatment of the corrected data, a one-way analysis of variance was done for each element-formula-level (native and spiked) combination to obtain components of variation within and between laboratories. S_o is the within-laboratory standard deviation; it represents the variability of measurements by one laboratory. CV_o is the coefficient of variation within a laboratory. The quantity S_L is the standard deviation representing the variability of laboratory averages with CV_L, the coefficient of variation. The reproducibility standard deviation, S_x, is derived from 2 quantities, S_o and S_L, and is calculated from the equation:

$$S_x = \sqrt{S_o^2 + S_L^2}$$

CV_x is the corresponding coefficient of variation.

S_L^2 is the "true" between-laboratory variance, i.e., it is the between-laboratory variance with the values S_o^2/r, representing within-laboratory variability, subtracted (r being the number of replicate readings per laboratory). Thus, it is possible to obtain a value of zero for S_L (if S_o is great) even though the laboratory averages differ, as is the case for Mg in the spiked Brand C ready-to-feed sample.

For the samples analyzed and the elements determined, most values for CV_x, CV_o, and CV_L were below 9%. For certain elements at elevated levels, such as Na, Ca, and K, most values for CV_o were less than 4% with many values in the range of 1–2%, indicating excellent within-laboratory repeatability.

One striking deviation from this pattern was for the Fe determination in Brand D powder at the native (unspiked) level. For this sample, the values of CV_o and CV_x were 63.9

Table 11. Concentration (μg/mL) of elements in blanks

Element	Laboratory					
	1	2	3	4	5	6
Ca	0.04	0.05, 0.05	0.23, 0.19	0.42, 0.13	0.31, 0.33	0.07, 0.07
Cu	ND[a]	ND	<0.05	ND	0.04, 0.03	ND
Fe	0.05	ND	0.03, 0.02	ND	0.04, 0.04	0.02, 0.01
K	0.96	ND	<300	1.14, 0.85	0.67, 0.72	ND, 0.07
Mg	0.01	ND	<0.5	ND	0.16, 0.17	0.06, 0.03
Mn	ND	ND	<0.01	0.01, 0.01	0.01, 0.01	0.001, 0.001
Na	0.23	0.04, 0.04	<30	0.56, 0.92	3.26, 2.66	ND
P	ND	ND	<0.5	ND	0.35, 0.40	ND
Zn	ND	ND	0.01, 0.01	0.01, 0.02	0.03, 0.02	0.004, 0.003

[a]ND = not detected.

and 66.3, respectively. The recovery for the spiked sample was also low, 79%. The values of CV_o and CV_x for the spiked samples were 12.8 and 14.6, respectively, somewhat lower than the values found for the native level but still considerably higher than those for Fe determination in other samples. The authors have no definite explanation for the discrepancy between this sample and the others, although the possibility of heterogeneity or contamination of the sample before being sent to the collaborators seems the most plausible.

Recoveries obtained on spiked samples were generally very good with most values ranging from 90 to 105%. The most notable exception to this, as previously discussed, was Fe in Brand D powder with a recovery of 79%.

A final area to be considered is the elemental concentration of the blanks. Each laboratory was to run 2 blanks along with the samples and report the concentrations as μg/mL. The values for the blanks are presented in Table 11. Sodium concentrations ranged from "not detected" in Laboratory 6 to 3.26 μg/mL for one blank in Laboratory 5. Similar results were found for K with a range from "not detected" to 1.14 μg/mL. Ca ranged from 0.04 to 0.42 μg/mL. The range of blank values for these 3 elements is not surprising in view of their ubiquitous nature. The variability of the blanks for these elements does not pose a problem for their determination in infant formulas as these elements are present in formulas at levels at least 100 times greater than the blank concentrations. Thus any contribution from the blank is trivial. Other elements were reported either as "not detected" or at levels so low as not to be of concern for their determination in infant formula.

Recommendation

It is recommended that the inductively coupled plasma emission spectroscopic method for the determination of Ca, Cu, Fe, Mg, Mn, P, K, Na, and Zn be adopted official first action.

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SYMPOSIUM ON LIQUID CHROMATOGRAPHIC ASSAY OF VITAMINS

97th Annual International Meeting of AOAC, October 1983

Liquid chromatography (LC) has become an effective tool in the assay of vitamins in nutritional products and foods. It offers alternative methodology to the more tedious chemical, spectrophotometric, and microbiological assays. Over the past 10 years, LC methods for determining oil- and water-soluble vitamins in fortified nutritional products and foods have proliferated. In recent years, much attention has been given to determining naturally occurring vitamins in foods.

The growth of new technology and its broad applications to vitamin analyses dictated the need for a symposium on LC assay of vitamins. The abundance of research on vitamins could not be presented in one symposium. Consequently, 2 sessions were presented by AOAC. Oil-soluble vitamins were discussed at the April 1983 AOAC workshop in Indianapolis. During preparation for the present symposium on water-soluble vitamins, it was apparent that all research on these vitamins also could not be discussed within a single symposium. Thus, the October symposium covered only the following: development of B-vitamins technology as illustrated by analytical changes undertaken to improve LC analysis for vitamins in fortified tablet products; discussion of problems encountered in adapting procedures for foods analyses; and new developments for testing vitamins traditionally assayed by microbiological methods (pantothenic acid and biotin) and testing vitamin B_{12} in tablet products.

LC has primarily been successful in the analysis of water-soluble vitamins in nutritional and pharmaceutical products, where they are typically fortified at 100% USRDA. Vitamins such as biotin, B_{12}, and pantothenic acid are difficult to measure because of either the low concentrations encountered or lack of an adequate chromophore for UV detection. Conversely, vitamins B_1, B_2, B_6, and niacin usually are found at higher levels, and each exhibits easily measurable absorbance at traditional wavelengths. Advances in LC methodology have occurred in the simultaneous analysis for these 4 vitamins. Recently, folic acid and ascorbic acid have received more attention. Analyses for these vitamins are complicated by their instability in solution because of reactions with oxygen or minerals.

Extension of LC methods to analyses for vitamins in food products, both fortified and unfortified, has been a primary goal of many investigators over the past 5 years. Even for fortified food products, existing LC methods have not always been directly applicable because of interferences from fatty acids and proteins. In addition, relatively low vitamin concentrations made isolation and detection difficult. In unfortified foods, the presence of multiple vitamin forms was an additional problem. For example, vitamin B_6 can be present as the free or phosphate forms of pyridoxal, pyridoxine, and pyridoxamine. Multiple forms of vitamins increased the need for better chromatographic separation and, in some cases, complicated vitamin isolation because of the differences in solubility of each form. Despite these factors, many effective methods have been developed using improved ion-pair techniques, dual column separation, and improved detectors.

The vitamin symposium addressed these problems and presented feasible procedures for assay of water-soluble vitamins. In designing the program, an attempt was made to include participants from industry, government, and academia so that their varied backgrounds would demonstrate different approaches to method development as well as identify different concerns on vitamin analyses in each area.

Following this introduction are 5 of the presentations. The information presented in the sixth speech was primarily covered in a previous publication: Holcomb, I. J., Lam, F. L., & Fusari, S. A. (1981) *Anal. Chem.* **53**, 607–609.

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Determination of Pantothenic Acid, Biotin, and Vitamin B_{12} in Nutritional Products

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Until recently, liquid chromatographic (LC) methodology for pantothenic acid, biotin, and B_{12} (cyanocobalamin) has been only marginally successful. These vitamins are difficult to determine by conventional LC techniques and UV detection at 254 or 280 nm, because either the chromophore is inadequate for detection or interference from co-eluting vitamins is overwhelming. Biotin and B_{12} are usually present in pharmaceutical products at concentrations 100–1000 times lower than other commonly occurring water-soluble vitamins. Co-extraction of all water-soluble vitamins results in gross interferences, especially in LC when the interfering vitamins co-elute with biotin or B_{12}. In addition, pantothenic acid and biotin are colorless in solution and do not exhibit strong UV absorption above 240 nm. As a result, they must be quantitated either by using a low UV wavelength for detection or by derivatizing the vitamin to obtain an adequate chromophore. A description of procedures for LC determination of pantothenic acid, panthenol, cyanocobalamin, and biotin in pharmaceutical products is presented. Pantothenic acid has been measured by using both a derivatization technique and low UV wavelength detection. Biotin has been quantitated by using low UV wavelength detection. The limitations of these techniques are also discussed. Chromatographic separation of cyanocobalamin is complicated by co-eluting vitamins such as riboflavin. It is detected by using the 546 nm wavelength where riboflavin does not interfere.

Pantothenic Acid and Panthenol

Pantothenic acid is usually found as the calcium or sodium salt in solid pharmaceutical preparations and as panthenol in liquid products. Pantothenic acid is colorless in solution, and does not have a UV chromophore to provide absorption above 230 nm; hence, it cannot be detected at the typical 280 or 254 nm wavelengths. Pantothenic acid must be measured at a low UV wavelength or after derivatization to a compound possessing a strong chromophore.

Combining acid hydrolysis and fluorescamine derivatization with liquid chromatography (LC) permits determination of panthenol in liquid pharmaceutical products (1) by using fluorescence detection. Aminopropanol, formed by the acid hydrolysis of panthenol, is derivatized with fluorescamine. The aminopropanol-fluorescamine complex is chromatographed on a C_{18} column with methanol–borate buffer (0.1M, pH 8.0) mobile phase.

In our laboratory, a modification of this procedure was used to determine panthenol in liquid multivitamin products. The basic procedure is to hydrolyze a sample containing approximately 1 mg panthenol 30 min at 85°C with 50 mL 0.5N HCl. After cooling, particulates are removed by centrifugation, then 1 mL supernate is mixed with 10 mL fluorescamine solution (0.4 mg/mL) and diluted to 25 mL with 1% sodium borate solution. The aminopropanol-fluorescamine complex is chromatographed on a μBondapak C_{18} column with an acetonitrile–0.1M ammonium acetate (18 + 82 v/v) mobile phase. Using fluorescence detection, the excitation wavelength is set at 390 nm and emission is between 475 and 490 nm. Figure 1 shows a typical chromatogram.

This procedure is limited to samples that contain at least 100 μg panthenol/g. The average recovery was 99% and precision was 2.9% RSD at 1.03 mg panthenol/g. The procedure is not adequate for vitamin stability studies without analyzing for aminopropanol separately. Excellent agreement was found when the LC method was compared with the microbiological procedure.

Pantothenic acid is more difficult to determine because of interferences during derivatization. Pantothenic acid hydrolyzes to β-alanine and pantoyl-lactone, and β-alanine can be derivatized with ninhydrin, 1,2-naphthoquinone-4-sulfonate, hydroxylamine-sodium hydroxide, 2,7-naphthalenediol, or 2,4-dinitrophenylhydrazine to produce colored compounds (2–7). These procedures are lengthy and can require extensive sample cleanup before the hydrolysis step because of various interferences from vitamin B_2, vitamin B_2-5-phosphate, niacinamide, zinc, copper, manganese, and molybdenum. Ion-exchange sample cleanup on Dowex 50-X4 (H^+ type) is required to remove these interferences.

We used the fluorescamine procedure for pantothenic acid analysis, and although minerals did not interfere, the method was not totally reproducible. The fluorescamine-alanine complex would periodically yield 2 chromatographic peaks, and the cause of this is uncertain.

Pharmaceutical products and vitamin premixes can be analyzed using a reverse phase LC technique (8) and low UV wavelength detection. Pantothenic acid is extracted from tablets or powdered premixes with 0.005M phosphate (pH 4.5) solution. Sonication thoroughly disperses the tablets to promote effective pantothenic acid extraction. Most of the undissolved material is subsequently removed by centrifugation and filtration. Pantothenic acid is separated from other water-soluble vitamins on an aminopropyl-bonded silica column with an acetonitrile–0.005M monobasic potassium phosphate (pH 4.5) mobile phase and detected at 210 nm.

Analysis using UV detection below 220 nm has inherent problems because of the limited number of common LC mobile phase solvents that have an appropriate UV cutoff and because of dissolved oxygen interference (9). Degassing the solvents used for the mobile phase via sonication under vacuum was very successful and much easier than heating or vacuum filtration alone.

A problem associated with using a phosphate–acetonitrile solvent system is the potential for phosphate precipitation in the LC column. A water–acetonitrile (90 + 10 v/v) column wash is very effective for maintaining column life. Our quality control group has used this procedure for over 18 months with no appreciable loss in column performance.

Figures 2 and 3 show typical chromatographic separations of pantothentic acid in a multivitamin-multimineral tablet and in a multivitamin tablet, respectively. Specificity was determined by taking the sample peak corresponding to the retention time of the pantothenic acid reference standard and recycling it through the column 4 times; no further separation occurred. This technique is useful for determining the presence of any co-eluting substances. The identity of the peak as pantothenic acid was confirmed by collecting the peak fractions from several sample injections, removing the acetonitrile portion of the mobile phase, and analyzing the peak material by microbiological assay (10).

A linearity study (ratio of concentration to peak area) showed complete linearity within the concentration range selected, 0.050–0.50 mg/mL, and a 0.998 correlation coefficient. Accuracy of the procedure was determined by spiking a placebo

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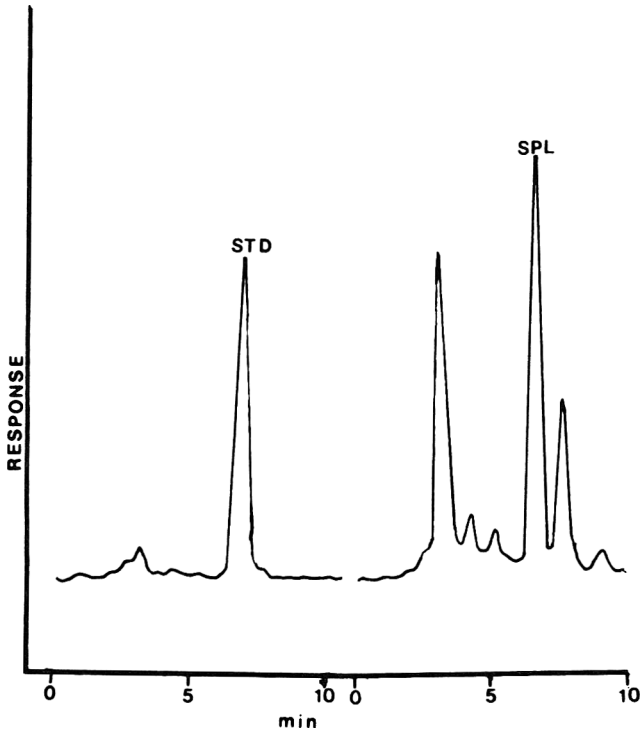

Figure 1. Representative LC chromatograms of aminopropanol complex formed from standard panthenol solution and sample extract solution containing panthenol. See text for conditions.

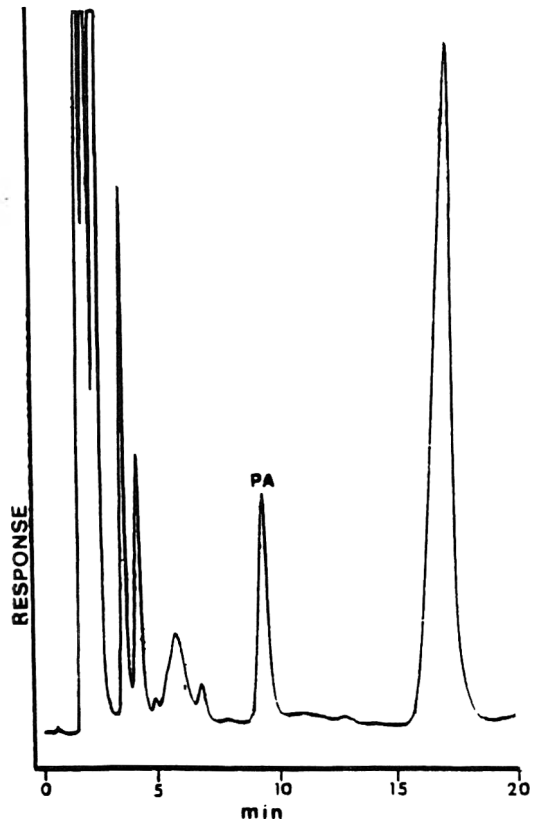

Figure 2. Chromatogram of multivitamin-multimineral tablet extract. PA is pantothenic acid. Conditions are given in text.

formulation and various samples with known concentrations of standard; precision was determined by analyzing 8 identical samples on different days. Quantitative recoveries (>95%) and good precision were obtained for multivitamin tablets, fortified yeast, vitamin premix, and raw material.

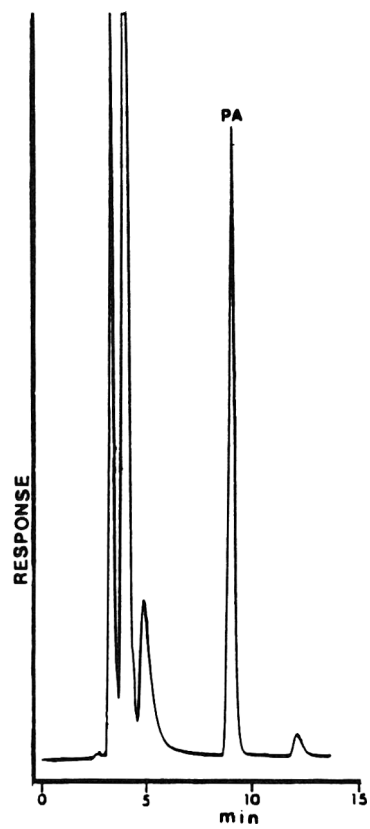

Figure 3. Chromatogram of multivitamin tablet extract. PA is pantothenic acid. Conditions are given in text.

Table 1 shows a comparison of results for the LC method described and the USP microbiological method for various commercial vitamin tablet samples. Low levels of calcium pantothenate (<3 mg/tablet) were more precisely analyzed by the LC procedure than by the USP microbiological method, and this may be related to the larger sample size required for the LC procedure, because any sample heterogeneity will have less effect with a larger sample.

This procedure was also used to analyze several commercial products for calcium pantothenate; Table 2 shows the results.

Two limitations of this method have been identified: First, the limit of sensitivity is approximately 1 mg/g sample. Although the method can be used to analyze most tablet products, further sample manipulation is necessary for analysis of most food products. Second, the aminopropyl column must be checked for system suitability, because of observed variations in performance of columns from different manufacturers.

Biotin

Biotin is one of the lesser known water-soluble vitamins. It has had limited use in fortified vitamin products, mainly because of its high cost. Biotin is found at 50% USRDA or less in many commercial products. Biotin is a coenzyme essential in amino acid metabolism and in maintenance of skin, hair, and nerves. It also has an important role in growth, decarboxylation of amino acids, and carbohydrate metabolism.

The microbiological assay using *Lactobacillus plantarum* is the most common analytical assay for biotin. Microbiological procedures require 18–24 h incubation in addition to sample preparation time to obtain final results. This is obviously not amenable to rapid quality control testing. Positive inter-

Table 1. Comparison of pantothenic acid results from USP method and LC method

Product[a]	Label, mg/tab.	Found,[b] mg/tablet (± SD) LC	USP
A	3.0	3.65 ± 0.075	3.73 ± 0.176
B	3.4	3.81 ± 0.099	3.88 ± 0.178
C	2.0	2.87 ± 0.041	2.66 ± 0.188
D	40.0	46.78 ± 0.823	47.0 ± 2.851
E	64.0	68.77 ± 2.077	67.84 ± 2.763
F	70.0	67.96 ± 1.990	66.47 ± 3.661
G	80.0	86.69 ± 1.830	87.41 ± 4.014

[a]A–C: multivitamin-multimineral tablets.
D–F: B-complex tablets.
G: stress formula B-complex tablet.
[b]Average of 5 determinations.

Table 2. Pantothenic acid found in commercial products by LC procedure

Product	Label, μg/tab.	Found, μg/tab.
A	20	27
B	20	29
C	10	14
D	10	12
E	2.5	3.1

Table 3. Comparison of biotin results from LC and microbiological methods

Product	Microbiol.	LC
B-Complex tablets	460 μg/tab.	450 μg/tab.
Vitamin premix 1	4.3 mg/g	4.0 mg/g
Vitamin premix 2	3.8 mg/g	4.5 mg/g

Table 4. Replicability and recovery results for LC determination of cyanocobalamin in various products and premixes

Product	Av. found[a]	Replicability, % RSD[b]	Rec., %
Vitamin concentrate	175.8 μg/g	3.8	95.3
Vitamin premix 1	1.95 μg/g	1.1	93.0
Vitamin premix 2	13.22 μg/g	5.0	100.0
B-complex	32.0 μg/tab.	1.3	96.0
Product A (with Fe)	7.2 μg/tab.	2.1	103.5
Product B	15.4 μg/mL	4.1	93.0
Product C	3.3 μg/tab.	6.8	90.3
Product D (for children)	2.1 μg/tab.	2.3	98.0

[a]Cyanocobalamin added at levels of 50, 100, 150, and 200% of theoretical.
[b]A homogeneous sample was divided into 5 portions and B_{12} was determined on 2 different days.

ferences due to the growth-stimulating compounds, oleic and aspartic acids, have been reported (11). We are not aware of any published LC methods for biotin in pharmaceutical or food products.

Our laboratory has an ongoing program to develop chemical procedures for vitamins contained in our tablet and food products. This report describes the preliminary investigation of the LC analysis for biotin in fortified tablet products. The procedure is currently being refined. Biotin does not have an adequate UV-absorbing chromophore; UV detection at typical wavelengths (254 or 280 nm) is not feasible. However, we did investigate low wavelength detection, as described for pantothenic acid analysis.

Biotin was extracted with a phosphate buffer solution and chromatographed on an aminopropyl column. Interferences from riboflavin and pyridoxine made quantitation difficult because these vitamins co-elute with biotin, and baseline resolution could not be obtained. Column variation was a problem because retention times were erratic.

We made various attempts to clean up the sample by gravity column chromatography. Sample cleanup columns containing bonded silica from various manufacturers were examined. A Sep-Pak C_{18} cartridge was effective, but biotin was not completely isolated from water-soluble riboflavin.

The current procedure for isolating biotin is applicable to preparations that contain at least 300 μg biotin. The samples are dissolved with sonication in 25 mL of pH 3.5 potassium phosphate, and centrifuged 5 min at 10 000 rpm to remove most particulates. A 2 mL aliquot is filtered through a 0.45 μm filter, placed on a C_{18} Sep-Pak column, then washed with 2 mL water followed by 3 mL phosphate buffer and 2 mL 5% acetonitrile–phosphate buffer. Biotin is eluted with 15% acetonitrile-phosphate buffer.

Chromatographic separation was performed on a Vydac HS C_{18} column (250 mm × 4.5 mm id), 5 μm particle size, using a pH 3.5, 0.01M KH_2PO_4–acetonitrile (90 + 10 v/v) mobile phase and detection at 230 nm. Figures 4 and 5 show typical chromatograms. A linearity study of biotin over the range 75–300 μg/mL gave a 0.989 correlation coefficient.

The Vydac column was the most suitable for this type of analysis. Other columns, such as Waters μBondapak and Merck Hibar did not demonstrate the efficiency required to separate biotin from riboflavin with the aforementioned mobile phase. It is likely that the column stationary phase loading is important in this type of separation.

Replicability and recovery data for biotin have been obtained for this procedure. Precision was determined by dividing a homogeneous sample into 8 fractions. These samples were tested on different days. Recovery was measured by spiking a placebo material at levels from 50 to 200% of theoretical value. Average recovery was 94.2% with a 5.08% relative standard deviation for B-complex tablets and vitamin premixes.

Selectivity of the procedure was studied by comparing LC results with microbiological results (12) (Table 3).

At this time, the LC procedure is applicable only to multivitamin products containing at least 150 μg biotin/tablet. However, the procedure is useful for premix testing, because testing time for this aspect of production can be decreased.

Apparent interferences from minerals lower the recovery of biotin from multivitamin and multimineral products. We are currently attempting to identify the interfering minerals. A concentration or derivatization step is needed so lower biotin levels can be determined.

Cyanocobalamin

Vitamin B_{12} (cyanocobalamin) contained in finished products and vitamin premixes is usually measured by the AOAC microbiological procedure (13). Raw materials can be determined spectrophotometrically as well (14, 15). Spectrophotometric methods, in addition to being limited in sensitivity, are susceptible to interferences from colored excipients.

Until recently, routine application of LC for assay of cyanocobalamin in pharmaceutical products had not been reported. Butte et al. (16) used dual column reverse phase chromatography to measure various cobalamin analogs in plasma. However, this procedure would be too intricate and time-consuming for routine product analysis.

The following procedure is applicable to the analysis of cyanocobalamin in pharmaceutical products, premixes, and raw material (17): A sample containing between 20 and 100 μg cyanocobalamin is extracted using either DMSO–water

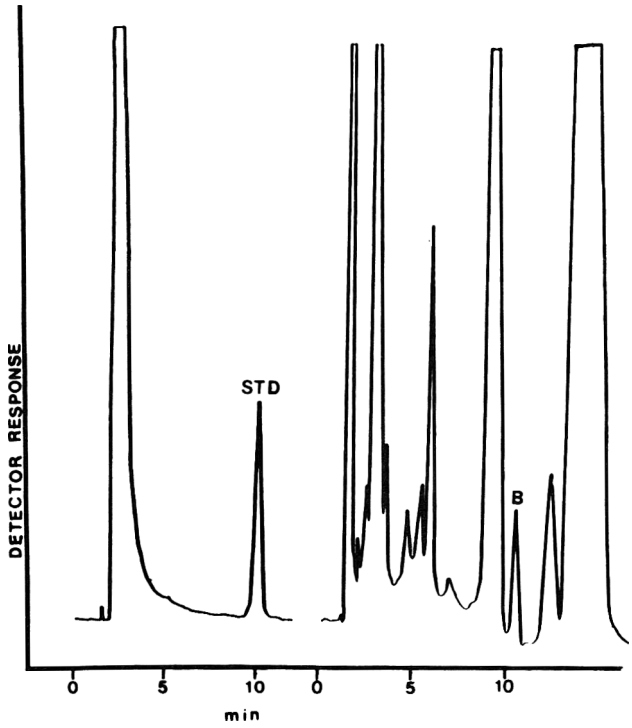

Figure 4. Biotin standard chromatogram and chromatogram obtained from multivitamin tablet extract. Peak B is biotin.

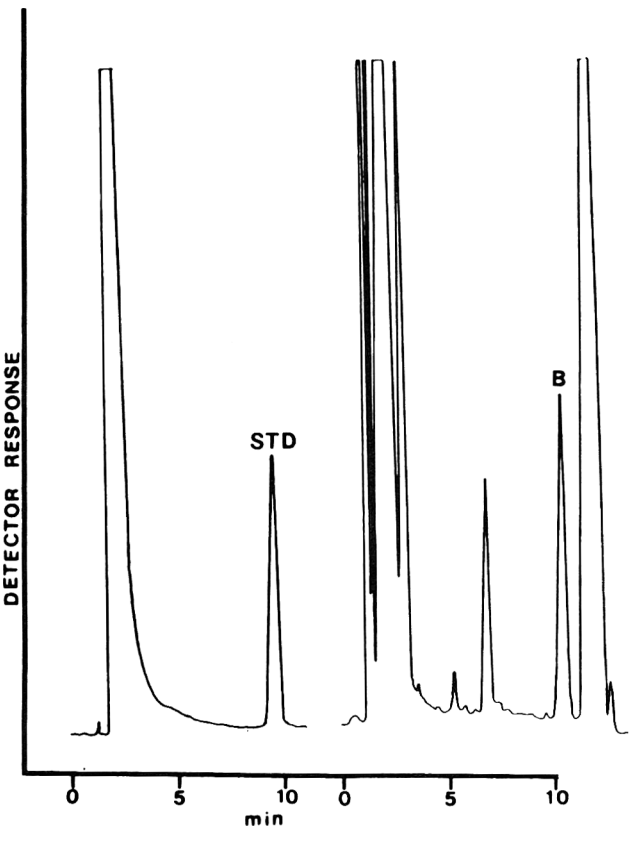

Figure 5. Biotin standard chromatogram followed by chromatogram obtained from vitamin premix extract. Peak B is biotin. Conditions are given in text.

(50 + 50 v/v) or 0.5% ammonium pyrrolidine dithiocarbamate (APDC) and 2% citric acid in DMSO and water (50 + 50 v/v). Samples that contain vitamin C and/or copper and iron ions require the latter extraction system to stabilize vitamin B_{12} in solution and to achieve complete recovery of the vitamin.

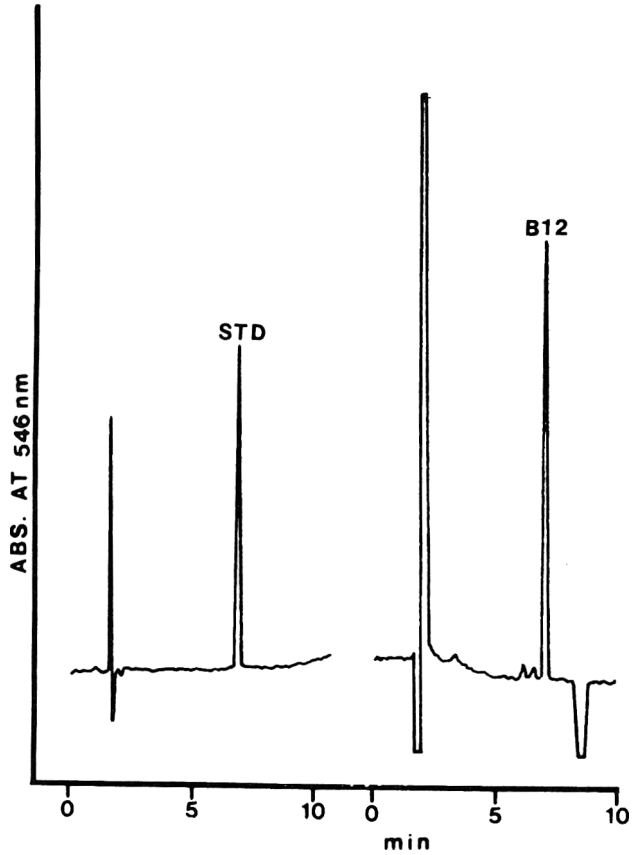

Figure 6. Chromatogram of cyanocobalamin standard and chromatogram of vitamin premix concentrate extract. Peak B_{12} is cyanocobalamin. Conditions are given in text.

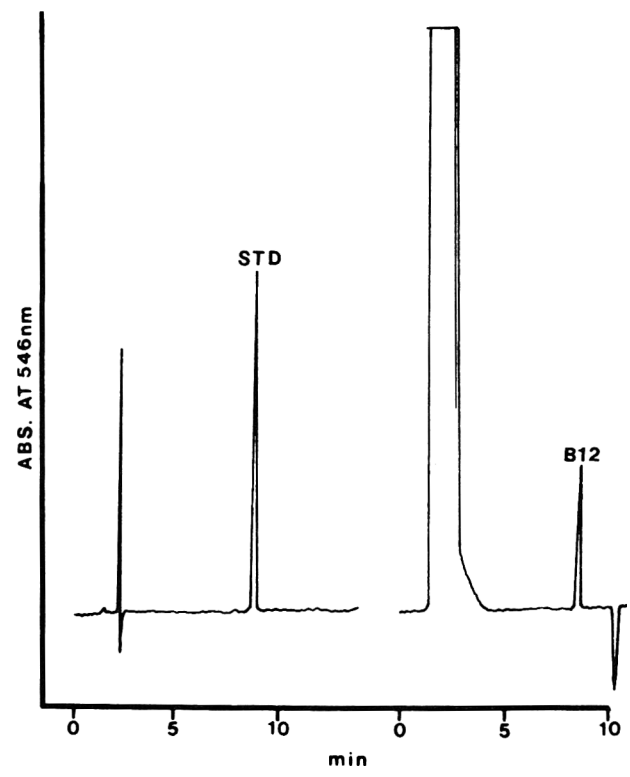

Figure 7. Cyanocobalamin standard chromatogram followed by chromatogram obtained from multivitamin-multimineral tablet extract. Conditions are given in text.

Table 5. Comparison of LC and microbiological assay results for cyanocobalamin

Product	LC	Microbiol.
B-complex	32.0 μg/tab.	32.6 μg/tab.
Product A	15.4 μg/5 mL	14.9 μg/5 mL
Product B (with Fe)	7.2 μg/tab.	7.6 μg/tab.
Multivitamin concentrate	179.6 μg/g	175.5 μg/g

Table 6. LC determination of cyanocobalamin in commercial products

Product	Label, μg/tab.	Found, μg/tab.
Product A	2.3	3.31
Product B	5	7.0
Product C	9	9.6
Product D	9.0	10.8
Product E	9.0	11.0
Product F multivitamin-multimineral	9.0	11.2
Product G multivitamin-multimineral	10	12.1
Product H enriched B-complex	30	35.2
Product I B_{12}	50	54.2
Product J balanced B-50	50	60.7
Product K B_{12}	250	255

Cyanocobalamin appears stable in the presence of ascorbic acid alone, but ascorbic acid with copper and possibly iron ions cause loss of cyanocobalamin (18). APDC and citric acid apparently complex with the metal ions, thereby enhancing B_{12} stability in solution. After the sample is shaken in the extraction solution 45 min at 55°C, the cooled sample is centrifuged to remove particulates. A portion of the supernate is filtered through a 0.45 μm filter before LC separation and analysis. Cyanocobalamin was separated from other water-soluble vitamins on a C_{18} μBondapak column (250 × 4.6 mm id) using a methanol–water gradient (15 + 85 to 50 + 50) over 15 min. Hydroxycobalamin does not co-elute with cyanocobalamin under these conditions.

Cyanocobalamin was detected at 546 nm rather than 278 or 361 nm wavelengths. Although the B_{12} absorption at 361 nm is about 3 times that at 546 nm, the chromatographic peak from co-eluting riboflavin overwhelms the B_{12} peak at 361 nm. Riboflavin is usually present at concentrations 100 to 200 times that of B_{12} in pharmaceutical products, but it does not absorb strongly at 546 nm and, therefore, does not interfere with baseline resolution of B_{12} (Figures 6 and 7).

A linearity study conducted with B_{12} standard concentrations from 0.50 to 50 μg/mL gave a 0.998 correlation coefficient. Table 4 shows the replicability and recovery results. Replicability was determined by splitting a homogeneous sample into at least 5 portions and testing for B_{12} on 2 different days. Recovery was measured by spiking a known quantity of cyanocobalamin into an actual sample or placebo. A placebo in this case is a specially prepared material containing all product components except cyanocobalamin. Cyanocobalamin was added at levels of 50, 100, 150, and 200% of the theoretical level.

Results from the LC procedure compared favorably with those obtained by microbiological assay (Table 5). The samples tested contained only cyanocobalamin and potentially hydroxycobalamin as a decomposition product. Thus, differences due to multiple cobalamin analogs were not possible, and any difference found would be due to method accuracy for cyanocobalamin.

The LC procedure was used to test several commercial vitamin products (Table 6). There was no analytical difficulty for any of the products listed; however, 2 products contained a colored material that interfered with the B_{12} analysis.

The basic limitation of this procedure is the low sensitivity to cyanocobalamin. As described for the method, samples must contain at least 0.8 μg cyanocobalamin/g product. To adapt this procedure to food products will require further studies on potential concentration techniques using Sep-Pak or Baker cleanup columns.

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Liquid Chromatographic Determination of Vitamin B-6 in Foods

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Chromatographic analysis for vitamin B-6 in complex samples imposes certain requirements on the analyst, who must extract completely the bound, unstable vitamers without loss, remove interfering compounds, and provide clean extracts for analysis. The analyst also has to contend with the problems inherent in all methods, such as sample collection, storage, preparation, and homogenization. However, chromatography provides a means of identifying and quantitating all forms of the vitamin, and thus provides the possibility of addressing the problem of the bioavailability of specific vitamers. It also allows automation, which is absolutely essential in coping with the large numbers of samples that are generated in areas such as quality control. These factors are all addressed here, and chromatographic results for various meat and other food products are presented to illustrate the variations in vitamin content that occur from sample to sample, the agreement with microbiological results, and that liquid chromatography (LC) has come of age in dealing with complex biological samples, such as food and food products.

A number of difficulties must be recognized and overcome by the analyst who uses liquid chromatography (LC) for the determination of vitamin B-6 in foods. Many of these problems are not unique to vitamin B-6 analysis, but are likely to be encountered in the analyses of other vitamins.

The problems can be grouped under 3 main headings: (1) collection, handling, and preparation of samples, (2) extraction and cleanup of complex samples, and (3) developing LC procedures that yield satisfactory results for food samples. We will also briefly comment on the problems encountered in addressing the question of bioavailability of the different vitamin forms. The data presented for pork, chicken, and tuna show that we found a surprisingly large variation in the vitamin B-6 content of supposedly similarly fed types of animals. Nutrient variability that occurs within a product or species has not been considered to any great extent by statisticians and nutritionists concerned with the nutrient content of a given diet.

Vitamer Structures, Forms in Food, Stability

There are 6 forms that exhibit vitamin B-6 activity: pyridoxamine (PM), pyridoxamine phosphate (PMP), pyridoxine (PN), pyridoxine phosphate (PNP), pyridoxal (PL), and pyridoxal phosphate (PLP). Figure 1 shows these structures as well as pyridoxic acid (4-PA), the metabolic byproduct of vitamin B-6. Of these six, in the free form PN is the most stable vitamer; the others are much more unstable, particularly in basic solution, with PLP the most unstable. When the vitamers (e.g., PMP or PLP) are bound to proteins, they are reasonably stable and can be stored for weeks without loss, particularly in muscle tissue.

Of the forms shown, it is generally agreed that PMP and PLP are the principal vitamers in meat, fowl, and fish; PN is the predominant form in fruits and vegetables. PLP is the predominant vitamer in fresh muscle tissue, but it is rapidly converted to PMP through transamination in cooking (1). Both PLP and PMP are bound to protein in muscle tissue, while there is little evidence for any PN binding, when it is present. In rice bran, however, it has been shown that PN is bound to a glucose molecule (2), and there are indications of unknown bound forms of PN and PL in orange juice (3).

Foods that contain approximately 5 μg vitamin B-6/g are considered good sources of this nutrient. In a complex food sample, however, the percentage of any given vitamer that is available to human digestion and absorption is questionable.

Bioavailability

The total amount of vitamin B-6 in a sample may not necessarily be a true measure of that available to a human. There are antagonists of vitamin B-6. The principal ones are either analogs, such as 4-deoxypyridoxine (4-DP), which compete with the coenzyme for binding sites on the apoenzyme, or hydrazides, which form hydrazones with PLP and inactivate the coenzyme (4–6). Thus, hydrazide drugs are particularly effective inhibitors because they form strong complexes with the aldehyde forms of the vitamin. Figure 2 shows typical structures of some antagonists.

Snell (7) has observed that, in biological systems, PL can complex with amino acids to form reaction products; Figure 3 shows some of these. Reaction products exhibit reduced or no vitamin activity in humans. Food processing can cause a loss of vitamin activity through the formation of reaction products that are unavailable to the organism. The cysteine–PL product shown in Figure 3 can be found in stored, heat-sterilized samples of milk. Gregory and Kirk have demonstrated that ε-pyridoxyllysine, a compound found in many food samples, has only partial vitamin activity and can be formed from vitamin B-6 during storage (8, 9).

A classic example that illustrates the effect of antagonists on vitamin B-6 bioavailability describes how the addition of linseed meal to a diet for chickens seriously inhibited their growth. The responsible antagonist was found to be 1-amino-D-proline (Figure 2), a secondary hydrazine which forms stable hydrazones with PL and PLP (10, 11). These hydrazones are, however, easily broken up with water treatment and drying. Thus, extraction followed by subsequent analysis would have indicated a diet adequate in vitamin B-6, when in fact, the vitamin, in a bound form, was mostly unavailable to the animals.

Table 1 lists the observed vitamin activity in animals for some known bound forms of the B-6 vitamers. Vitamin activity is expressed as a percent of the activity of the unbound form. Thus, to accurately report the bioavailable amounts of B-6 in a diet, the extraction procedures used must mirror those of the body's digestive and absorption systems, a truly formidable job for the analyst.

LC Analytical Systems

Several LC systems can successfully separate and quantitate all the forms of vitamin B-6 in foods. We present just 2 examples here: an anion-exchange procedure, and an ion-pairing reverse phase separation. Figures 4 and 5 show chromatograms of standard samples for these 2 completely different analytical systems. Details of the anion-exchange separation are given in the literature (13); the reverse phase procedure is a modification by Feldstein and Gregory (14) of

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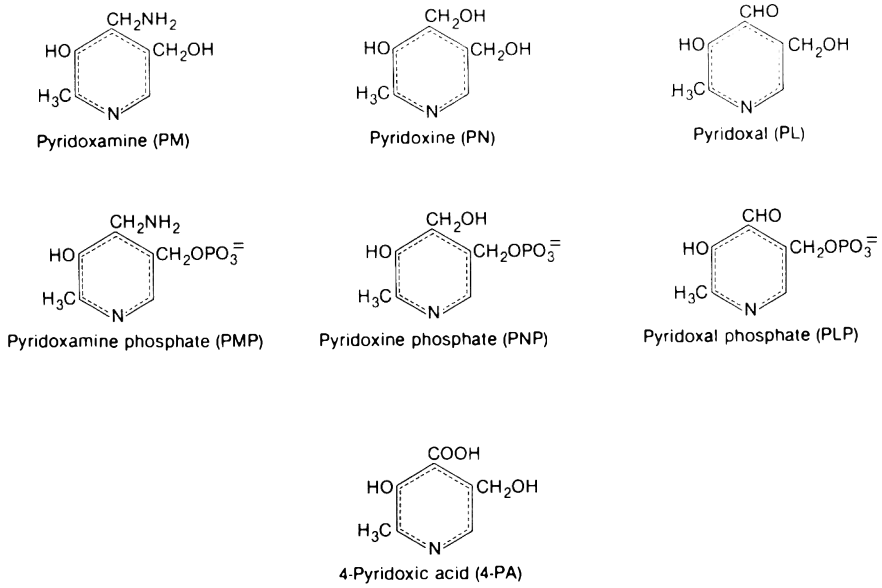

Figure 1. Molecular structures for the B-6 vitamers and their metabolic product, 4-pyridoxic acid.

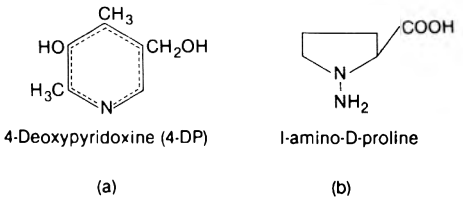

Figure 2. Antagonists of vitamin B-6. (a) an analog, (b) a hydrazine found in linseed meal.

an earlier separation by Tryfiates and Sattsangi (15). Both systems are capable of handling complex food samples after extraction and cleanup, and the systems are fully automated.

Each analytical scheme has certain advantages and disadvantages. The anion-exchange procedure uses a resin with a polystyrene-divinyl benzene co-polymer backbone. Such resins are particularly tough and stable, and have long lifetimes (5–6 years). Elution is isocratic in this procedure and, hence, column regeneration is not required after each run. The disadvantage of this anion-exchange method is the inefficiency caused by the relatively long (60 min) elution times that are required. Unfortunately, the resin manufacturer must have changed its production process, because the latest lots of this resin do not have the same characteristics as earlier lots, and are unsatisfactory. The reverse phase procedure, on the other hand, is much more efficient and separations are achieved in half the time required for the anion-exchange procedure. Elution, however, is not isocratic so column regeneration after each run is necessary. Column lifetimes are about 6 months to 1 year.

At present, there are no known gas chromatographic procedures to analyze for all 6 vitamers, mainly due to the difficulty of forming volatile derivatives of the phosphate vitamers (16). Cleanup procedures also cause problems.

Automation

Automation is a necessity today because large numbers of samples require analysis. LC methods are easily automated with present-day technology. Figure 6 illustrates the automation of the anion-exchange method mentioned earlier (17); an inexpensive microprocessor has been used to trip relays which, in turn, switch valves at predetermined times. As shown in Figure 6, the position of the valves determines the nature of the column stream, the sample to be injected, and whether the solvent will pass through 1 or 2 columns. The microprocessor is also used to switch the excitation and emission wavelengths of the fluorescence detector at given times. This system illustrates all that is needed for automation: *(1)* the ability to trip external relays, and *(2)* wavelength control of the detector.

At present, automation can be even easier than the above scheme. Some currently available automatic injection samplers have incorporated relay controls, as well as automatic start controls for the detector and integrator. There are also detectors that can be pre-programmed to switch wavelengths at specified times. These advances in instrumentation eliminate the need for any external microprocessor.

Extraction and Cleanup Procedure

In the extraction of vitamin B-6 from foods, the analyst is attempting to recovery unstable organic molecules at concentrations of 1 ppm or less from an organic matrix. An acid extraction is usually used for vitamin B-6 analysis. Early extraction procedures, using HCl in conjunction with autoclaving, have generally been replaced by procedures that use trichloroacetic, perchloric, tungstic, metaphosphoric, or sulfosalicylic (SSA) acid (16). These acids do not destroy the phosphorylated forms of the vitamin. The selected extraction procedure must be compatible with the final analytical procedure to yield relatively clean extracts, free of interfering compounds. The recently developed SSA method (18) yields clean extracts for analysis, and is compatible with both the anion-exchange and reverse phase analytical procedures mentioned earlier. Figures 7–10 show chromatograms obtained with the SSA extraction of a variety of biological samples (18–20). Although these chromatograms resulted from using the anion-exchange procedure, similar, clean chromatograms are also obtained with the reverse phase procedure (13). The chromatograms shown are free of interfering compounds. Recoveries range from 95–100% for all samples. SSA extraction does not work for all types of food samples, as shown by the interferences in Figure 11, a chromatogram of a green pepper extract.

Table 1. Activity of some bound forms of vitamin B-6[a]

Investigator	Food	Bound form	Activity(%)
Yasumoto et al. (2)	rice bran	PN-glucoside	100
Kratzer et al. (10)	linseed meal (chicken feed)	PL-hydrazone	0
Nelson et al. (3)	orange juice	PN, PL-90% bound, PM-20% bound, unknown-MW <3500	40
Bernhart et al. (12)	milk	PL-cysteine	20
Gregory & Kirk (8, 9)	formulated cereal	ε-pyridoxyllysine 50% bound after storage	60

[a]Vitamin activity expressed as % of activity of unbound form.

Table 2. Vitamin B-6 content of separable lean pork loin samples

		Vitamer content, nmol/g[a]					
Packer	Grade	PMP	PN	PLP	Total	CV, % (total)	Range (total)
A	1	4.4 ± 0.7	1.0 ± 0.4	27.0 ± 8.0	32.1 ± 7.1	22	19–49
	2	4.3 ± 0.9	1.5 ± 1.0	25.0 ± 6.0	31.9 ± 4.5	14	26–40
	3	4.7 ± 1.3	1.0 ± 0.5	28.8 ± 9.1	35.0 ± 7.7	22	23–47
Av.		4.5 ± 1.0	1.2 ± 0.6	26.9 ± 7.7	33.2 ± 6.6	20	
B	1	4.5 ± 0.8	0.86 ± 0.26	24.7 ± 5.7	30.2 ± 6.0	20	19–37
	2	3.7 ± 1.1	1.1 ± 1.3	23.3 ± 6.2	29.2 ± 4.0	14	24–37
	3	4.1 ± 1.2	1.4 ± 0.6	25.7 ± 6.1	31.6 ± 6.4	20	25–32
Av.		4.1 ± 1.0	1.2 ± 0.7	24.6 ± 6.0	30.4 ± 5.4	18	

[a]Wet basis, mean ± SD. PMP = pyridoxamine phosphate, PN = pyridoxine, PLP = pyridoxal phosphate. Ten samples analyzed for each grade.

Table 3. Vitamin B-6 content of raw separable lean chicken (roasters)

		Vitamers found, nmol/g[a]				
Producer	Part	PMP	PLP	Total	CV, % (total)	Range (total)
A	Breast	3.73 ± 1.25	44.01 ± 15.26	47.74 ± 15.31	32	32–67
	Thigh	4.33 ± 0.48	14.17 ± 2.95	18.50 ± 3.10	17	17–23
	Leg	3.99 ± 0.55	12.05 ± 3.56	13.63 ± 2.13	16	11–16
B	Breast	4.63 ± 1.28	42.71 ± 9.38	47.35 ± 8.96	19	36–57
	Thigh	4.28 ± 0.41	15.50 ± 2.15	19.78 ± 2.42	12	17–22
	Leg	5.21 ± 0.87	16.83 ± 2.74	21.73 ± 2.42	11	18–24

[a]Wet basis, mean ± SD. PMP = pyridoxamine phosphate, PLP = pyridoxal phosphate. Five samples analyzed for each part.

Sample Handling—Collection, Homogenization, and Storage

The procurement of a representative food sample is most often a difficult task. It would be difficult, for example, to obtain a sample of pork loin representative of what is consumed by the general population. The selection of such a sample for analysis involves a study of diets, major production sources, selection of grades of meat, marketing analyses, and consumption patterns. Unless an extensive statistical study is done for each of these factors, an enormous number of samples would have to be analyzed to obtain reliable, representative data (21). Because such capabilities are often beyond the scope of most laboratories, analysts usually ignore these factors. However, one should consider sampling factors when investigating the nutrient composition of a food product.

Sample uniformity is usually not a problem for liquid samples. The nonhomogeneity of solid samples originally caused problems until the appearance of the Robot-Coupe and other food processors. Sample homogenization using these processors provides the desired uniformity for vitamin B-6 analysis. For example, it would be possible to obtain a uniform, 1 or 2 g aliquot for analysis from a food sample originally weighing 250 g.

After homogenization, some samples, such as muscle tissue and fortified breakfast foods, can be frozen and stored for months without appreciable loss of vitamin content (19, 20). Liver samples, on the other hand, are unlikely candidates for storage, on the basis of our rat liver tissue analyses (20), where differences in sample handling produced variable results. Specific protocol for sample handling and storage must be developed for each new class of products; in some cases, immediate analysis may be warranted.

Vitamin Content and Variation in Some Meat and Fish

LC has been used to determine vitamin B-6 content of various food samples, and these values have been reported in the literature (14, 20 plus references cited therein). In the results presented here, we were concerned not only with the forms and amounts of vitamin B-6 in the different meat and fish products, but also in the variations observed from carcass to carcass within species and source.

Fresh pork, fresh chicken, canned tuna, and fully cooked, smoked hams were chosen for analysis. Raw pork center loin samples from carefully selected carcasses were obtained from 2 geographically dispersed packing plants and were prepared as previously described (21). Two brands of fresh chicken

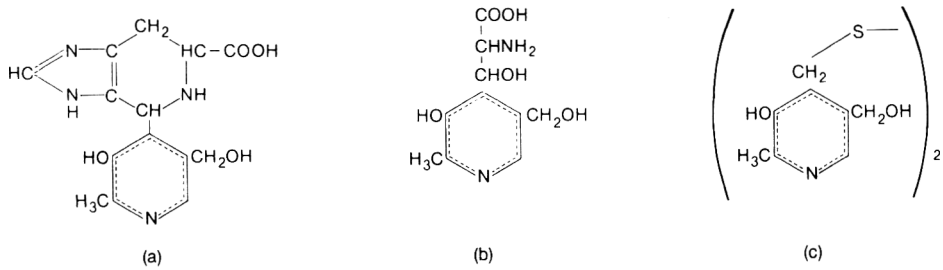

Figure 3. Reaction products between pyridoxal and (a) histidine, (b) glycine, and (c) cysteine.

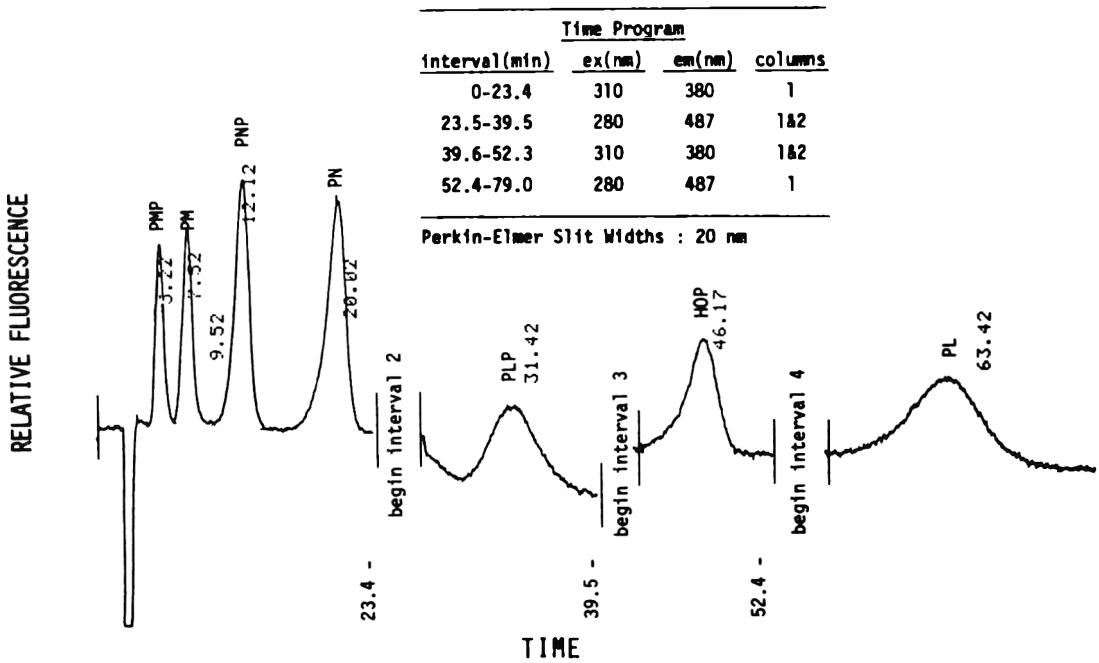

Figure 4. Chromatogram of analytical separation of vitamers with anion-exchange column. 3-Hydroxypyridine (HOP) used as internal standard. See Figure 1 for vitamer names.

roasters were selected locally and dissected into the usual retail parts. The breasts, thighs, and legs were skinned, boned, and homogenized, as described under *Sample Handling* preparation. All samples were frozen before analysis. The different types and brands of canned tuna were selected locally, drained of oil or water, and homogenized before analysis. Smoked hams were obtained from 4 different geographically dispersed packers, fabricated into shank and butt portions, cooked to an internal temperature of 140°F, and the cooked portions were composited. Each composite represented 6 portions collected at the same plant on the same day. The samples were frozen until analysis.

Table 2 gives results for the vitamin B-6 content of raw pork loins. Each sample analyzed represents the separable lean from a whole loin from a different carcass. Duplicate analyses on the same sample agreed within 5%. Analysis of variance indicates that there is no significant difference between grades or packing plants. It is surprising, however, to find such a large coefficient of variation (CV) and range of values for the same cut from carcass to carcass. Trace metal and other nutrient analyses also indicated surprisingly large variation between carcasses (21). The observed variation is not due to the methodology; the microbiological results discussed later agreed with the LC results. A large variation between carcasses presents a problem for the statistician who might be interested in determining the probability of finding a given amount of vitamin B-6 in a particular pork loin sample.

Table 3 presents data obtained for the vitamin B-6 content of different chicken parts from 2 producers. Again, each sample represents a different carcass. The CV for duplicate analyses of the same sample is less than 3%. There is a definite difference in the vitamin content of chicken breast vs thigh or leg, with the former containing significantly more vitamin B-6 than the latter—an observation previously reported (1). For breast and thigh, there is no significant difference in vitamin content between producers, while the *t*-test does indicate a significant difference for legs. This last result may be because sampling was limited. Again, the CV is larger than expected, particularly for breast meat across carcasses.

Table 4 shows the vitamin B-6 content of different types of canned tuna. The sixteen samples for each type comprise 4 samples from 4 different producers. There was no significant variation between samples from the same producer or between samples from different producers. Also, there is no significant difference in vitamin B-6 content among different types. It is interesting to note that CV values are less for tuna than for either fresh pork or chicken, despite the inherent variability in the tuna-canning process.

Table 5 shows vitamin B-6 content for high salt- and low salt-cured pork samples. We found no significant difference

Table 4. Vitamin B-6 content of canned tuna

Type	Vitamers found, nmol/g[a] PMP	PM	PN	PLP	Total	CV, % (total)	Range (total)
Chunk light, water pack	10.72 ± 1.29	10.80 ± 1.58	0.44 ± 0.31	0.68 ± 0.16	22.64 ± 1.27	6	21–24
Chunk light, oil pack	9.66 ± 0.22	6.92 ± 1.65	0.29 ± 0.59	0.95 ± 0.34	19.51 ± 1.92	10	18–23
Solid white, water pack	11.36 ± 1.81	9.40 ± 2.43	0.12 ± 0.14	0.95 ± 0.42	21.86 ± 2.55	12	18–24
Solid white, oil pack	11.17 ± 0.16	6.79 ± 1.87	—[b]	1.79 ± 1.00	18.95 ± 1.94	10	17–29

[a]Wet basis, mean ± SD. PMP = pyridoxamine phosphate, PM = pyridoxamine, PN = pyridoxine, PLP = pyridoxal phosphate. Sixteen samples analyzed for each type.
[b]None detected.

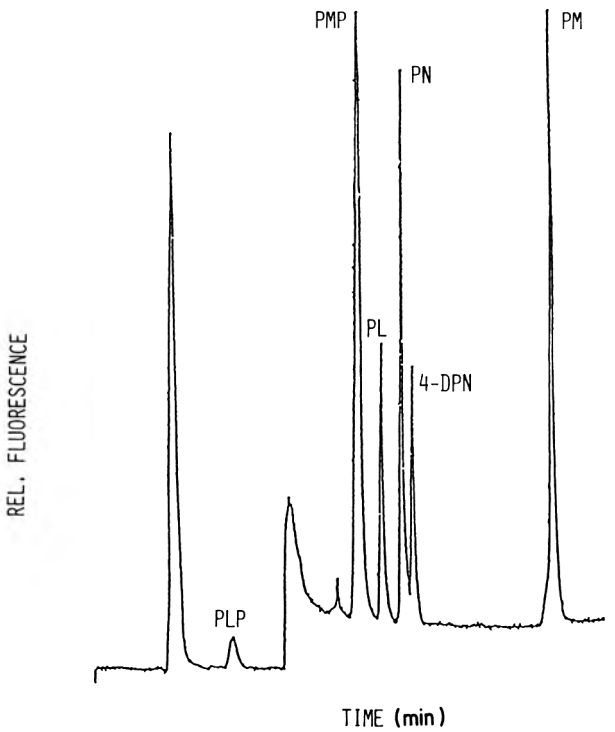

Figure 5. Chromatogram of analytical separation of vitamers with ion-pairing, reverse phase procedure. Chromatogram supplied by Feldstein and Gregory (14).

Table 5. Vitamin B-6 content of cured pork

Type of curing[a]	Vitamers found, nmol/g[b] PMP	PN	Total	CV, % (total)	Range (total)
High salt	11.57 ± 1.28	1.66 ± 0.36	13.26 ± 1.23	9	11–15
Low salt	11.01 ± 1.53	1.68 ± 0.47	12.69 ± 1.83	14	11–15

[a]Each sample is a composite of 6 individual hams roasted to an internal temperature of 140°F and dissected to form samples of separable lean. Ten samples analyzed for each type.
[b]Wet basis, mean ± SD. PMP = pyridoxamine phosphate, PN = pyridoxine.

Table 6. Cooking loss of vitamin B-6 in chicken[a]

Sample	Raw[b], nmol/g	% Loss Microwave	Conventional
1	114	20	14
2	102	3.5	5.2
3	130	18	29
4	132	5.0	24
5	—[c]	—	—
6	102	9.9	6.4
7	148	3.6	22
8	129	6.5	34
9	113	18	7.6
10	122	14	11
Av.	136 ± 22	10.9 ± 6.7	17.0 ± 3.5
CV, %	16	61	21

[a]Data from Wing and Alexander (22).
[b]Freeze-dried samples.
[c]Excluded result.

between the high and low salt-curing methods. Although data are not shown, we found no significant difference between butt and shank ends. No trace of PLP was found in these cured pork samples, even though Polansky and Toepfer (1) reported a significant amount of this vitamer in their study. CV values for cured pork analyses are similar to those for canned tuna.

Variability in Loss of Vitamin B-6 in Cooking

Preliminary data from our laboratory indicate a variability in percent loss of vitamin B-6 in cooking even with carefully controlled, identical cooking methods. We do not have enough data, however, to warrant statistical analysis. The data of Wing and Alexander (Table 6) (22), infer quite a variation in cooking loss from sample to sample. The variation in cooking loss added to the variation in vitamin content among raw samples makes it difficult to calculate the probability of obtaining a given amount of vitamin B-6 from a serving of chicken.

Comparison of Microbiological and LC Results

Chromatographic results normally agree well with microbiological results for most food samples. Gregory and coworkers (8, 23, 24) have made numerous comparisons of data obtained from their LC procedures with those obtained from microbiological methods. Their conclusions confirm the general validity of the chromatographic procedure. In fact, liquid chromatography is often superior to microbiological assays in both precision and ease.

The LC results for raw pork samples given here have been spot checked against the microbiological procedure by Polansky (25). The results for 10 of the samples are shown in Table 7; the comparison of methods shows satisfactory agreement. Table 8 shows the comparison of our mean values for chicken, tuna, and cured ham with those composite values reported by Polansky and Toepfer (1). For tuna and tuna liquid, the results are similar for both total and individual vitamer content. For chicken, the results for total content agree satisfactorily, but there is disagreement about the number and amount

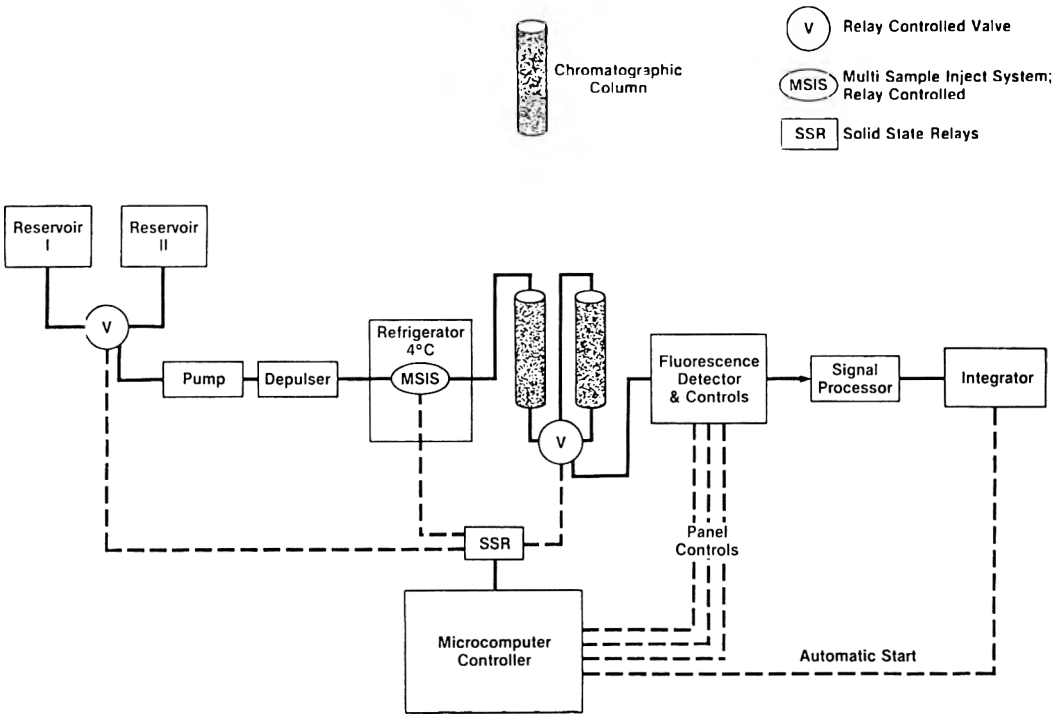

Figure 6. Diagram for automation of anion-exchange separation with microcomputer control.

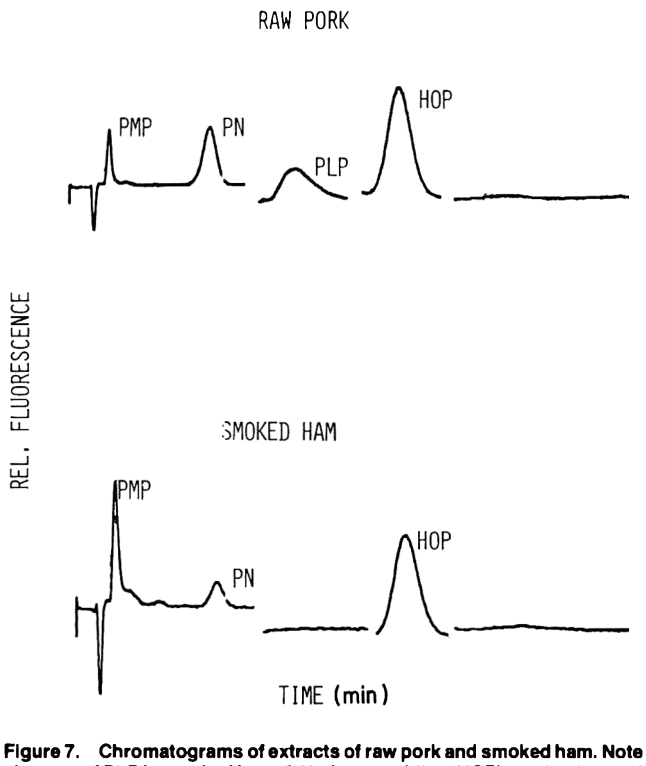

Figure 7. Chromatograms of extracts of raw pork and smoked ham. Note absence of PLP in smoked ham. 3-Hydroxypyridine (HOP) used as internal standard. See Figure 1 for vitamer names.

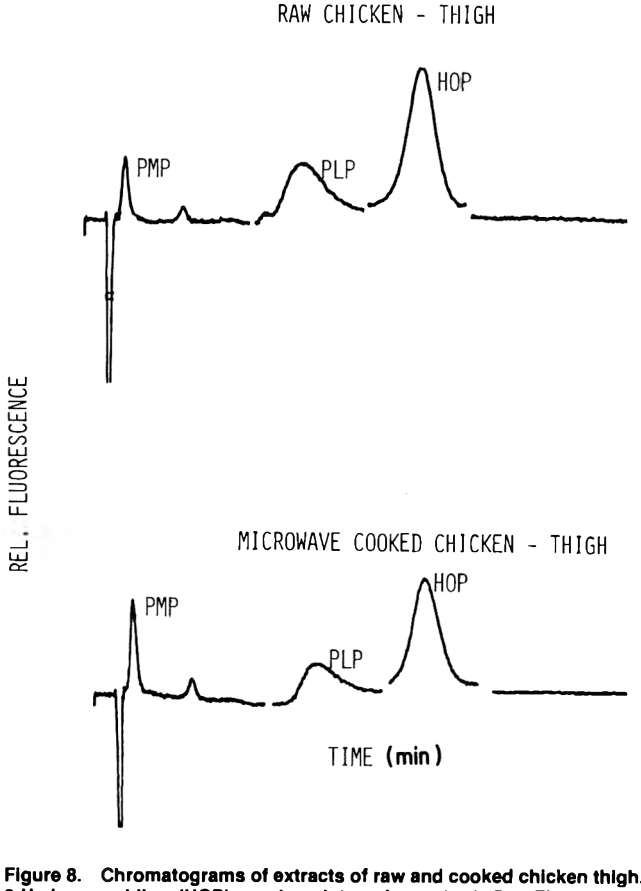

Figure 8. Chromatograms of extracts of raw and cooked chicken thigh. 3-Hydroxypyridine (HOP) used as internal standard. See Figure 1 for vitamer names.

of individual vitamers present. There is a definite discrepancy in the case of cured ham. As noted earlier, we failed to detect any trace of the aldehyde forms of the vitamin (PL and PLP), while Polansky found a significant amount (PL). Polansky kindly analyzed a few of our cured ham samples and obtained values which agreed with her published values. The reason for this discrepancy is not clear at the present time.

Conclusions

Our experience in vitamin B-6 analysis indicates that, with sufficient care in sample handling and extraction, LC techniques have advanced sufficiently to be used routinely in food

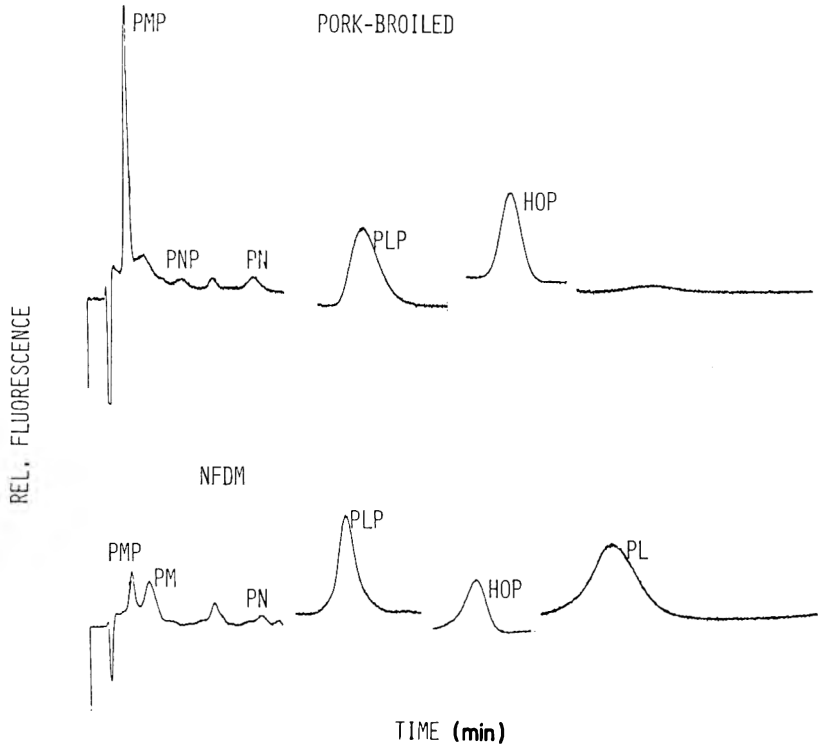

Figure 9. Chromatograms of extracts of broiled pork and nonfat dry milk. 3-Hydroxypyridine (HOP) used as internal standard. See Figure 1 for vitamer names.

Table 7. Chemical and microbiological assays of 10 raw pork samples

Sample	Total vitamin B-6, nmol/g[a] Chemical	Microbiological	Abs. diff.,[b] %
1[c]	9.3 ± 1.6	9.6 ± 1.1	3.2
2	22.4 ± 0.4	25.1 ± 1.4	11.3
3	35.4 ± 1.3	37.1 ± 1.7	4.7
4	29.0 ± 1.4	29.8 ± 1.5	2.7
5	37.4 ± 0.6	33.7 ± 1.4	10.4
6	36.9 ± 0.4	36.4 ± 1.2	1.4
7	32.1 ± 0.8	34.1 ± 2.7	6.0
8	40.2 ± 2.0	38.1 ± 1.6	5.4
9	31.9 ± 1.0	32.7 ± 2.9	2.5
10	34.1 ± 1.3	31.9 ± 1.1	6.7
Av. (2–10)	33.3	33.2	

[a]Wet basis, mean ± SD.
[b]Absolute difference of means for each sample times 100 divided by the average value.
[c]Pork fat back.

analysis and in quality control checks. Problems still exist, such as in the case of cured ham, but the outlook for LC analysis is promising. Quality control checks are important in infant formula processing, because formulas are normally the sole nutrient source in the early months of life. Figure 12 shows a typical chromatogram for an unknown infant formula. The determined amount of vitamin B-6 in this sample agreed exactly with that added by the manufacturer.

The great advantage of chromatographic procedures is that they are capable of detecting and quantitating all forms of vitamin B-6 in biological samples. Thus, we can at least begin to address the problem of the bioavailability of the different forms. This is by no means a simple job, because evidence must show whether extraction procedures are recovering amounts of the different vitamers that are available to the human digestive and absorption processes.

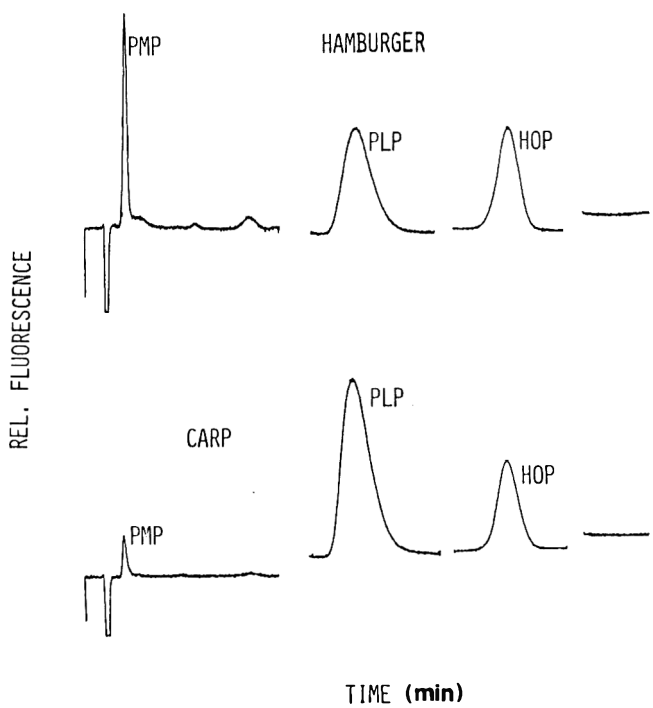

Figure 10. Chromatograms of extracts of raw hamburger and carp. 3-Hydroxypyridine (HOP) used as internal standard. See Figure 1 for vitamer names.

It is also becoming evident, as more food samples are being analyzed, that the variation in vitamin content between samples of the same type is quite large, as is the variation in the loss of the vitamin in cooking. These variations make it difficult to determine the probability that an individual will receive an adequate amount of vitamin B-6 from a given diet, and

Table 8. Vitamin B-6 content of several commodities, LC vs microbiological results

		Vitamers, nmol/g[a]							
		LC[b]				Microbiological[c]			
Meat	Cut	PM & PMP	PN	PL & PLP	Total	PM	PN	PL	Total
Chicken	breast	3.86	—[d]	43.36	47.54	7.76	2.60	30.4	40.76
	leg & thigh	4.72	—	14.31	18.95	6.99	1.60	10.7	19.19
Tuna	meat	21.14	0.30	1.50	22.3	20.6	0.30	1.50	22.4
	liquid (water)	25.23	0.36	0.28	25.9	24.3	0.36	1.32	26.0
Cured ham	leg	11.18	1.56	—	12.73	13.86	1.95	3.12	18.93

[a]PM = pyridoxamine, PMP = pyridoxamine phosphate, PN = pyridoxine, PL = pyridoxal, PLP = pyridoxal phosphate.
[b]Present work.
[c]Ref. 1.
[d]None detected.

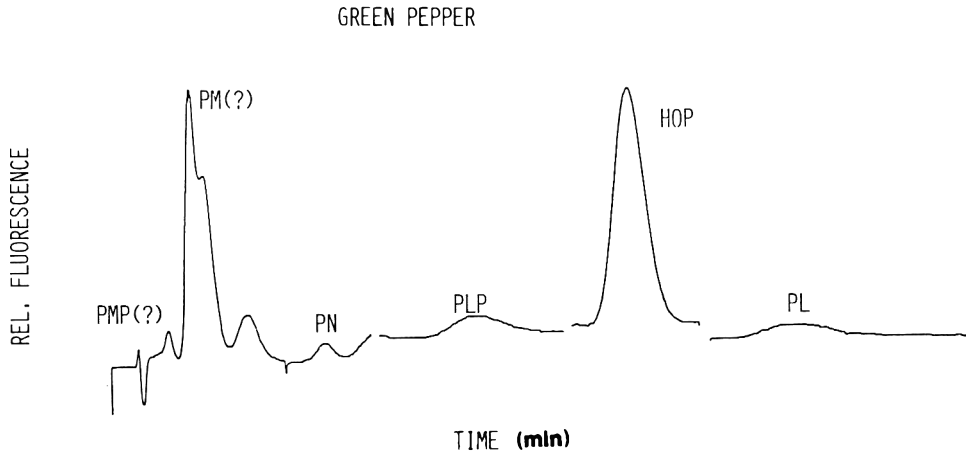

Figure 11. Chromatogram of extract of green pepper. 3-Hydroxypyridine (HOP) used as internal standard. See Figure 1 for vitamer names.

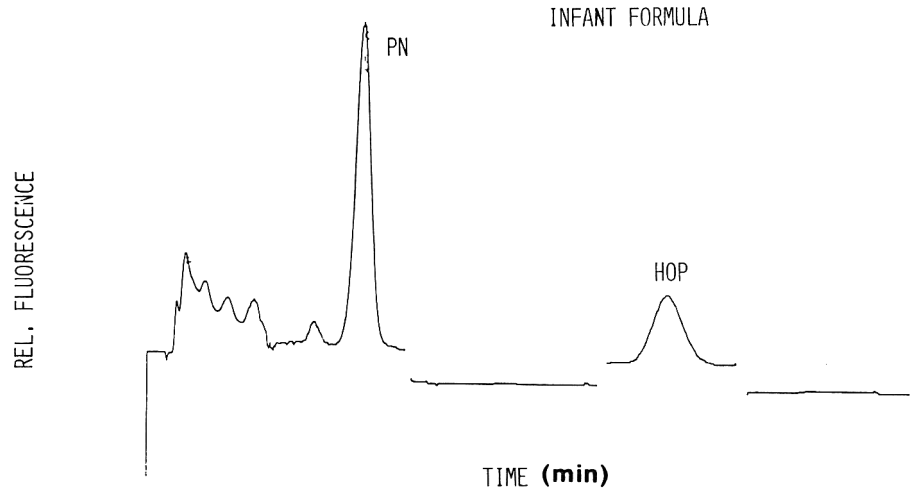

Figure 12. Chromatogram of extract from an instant baby formula. 3-Hydroxypyridine (HOP) used as internal standard. See Figure 1 for vitamer names.

add a new dimension to meeting nutrient requirements by dietary measures. We believe this is going to be a difficult undertaking, and we foresee the need for a large amount of additional data on food as it is eaten by the general public.

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Liquid Chromatographic Assay of Ascorbic Acid, Niacinamide, Pyridoxine, Thiamine, and Riboflavin in Multivitamin-Mineral Preparations

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A simple and rapid method is presented for the liquid chromatographic assay of ascorbic acid, niacinamide, pyridoxine, thiamine, and riboflavin from a single chromatogram. Ion-pair chromatography with a reverse phase C_{18} cartridge in a radial compression system is used. Quantitation is excellent with a total analysis time of less than 20 min. A mobile phase of methanol–water (15 + 85) (0.005M heptanesulfonic acid) with 0.5% triethylamine at pH 3.6 and a flow rate of 2.0 mL/min gives the most satisfactory separation of the 5 water-soluble vitamins. By using 2 detectors in series set at different wavelengths and sensitivities, all 5 vitamins, with peak heights on scale, can be measured from a single injection; peak elution order is ascorbic acid, niacinamide, pyridoxine, thiamine, and riboflavin. Ascorbic acid is measured at 254 nm and the other vitamins, at 280 nm. The amount of amine modifier in the mobile phase is critical to the separation of niacinamide and pyridoxine. Recoveries of 5 water-soluble vitamins from spiked placebos were in the range of 98.2–102.0%. Confidence limits, ± 3 SD, were in the range of 1.0–5.4%. Overall, the results obtained using the liquid chromatographic method show excellent agreement with manual methods and automated analysis.

The determination of water-soluble vitamins in multivitamin-mineral preparations is a routine quality control assay. However, the USP methods are tedious and time-consuming, and current automated methods, although fast and reliable, can assay only a single vitamin in each run. Another disadvantage is frequent interference from excipients. Hence, the development of a rapid and reliable method for the simultaneous liquid chromatographic (LC) assay of water-soluble vitamins is necessary.

A number of papers on this subject had been published when we initiated our project in 1979. Two or more vitamins had been assayed simultaneously by chromatographic techniques such as ion-exchange (1), liquid-solid (2), reverse phase (3–5), and ion-pair (6) chromatography. Meanwhile, others (7, 8) reported the LC assay of 4 different vitamins: niacinamide, pyridoxine, thiamine, and riboflavin from a single injection. Among these different approaches, we were most interested in the ion-pair reverse phase LC method using a C_{18} cartridge with a radial compression system. This paper presents the application of the developed method to the analysis of ascorbic acid, niacinamide, pyridoxine, thiamine, and riboflavin in several commercial multivitamin-mineral preparations.

Part of this work was presented in the Anachem Symposium, Wayne State University, Detroit, MI (October 1980).
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This paper was part of the symposium "Instrumental Methods for the Analysis of Vitamins" which was held at the 97th Annual International Meeting of the AOAC, Oct. 3–6, 1983, at Washington, DC.

METHOD

Reagents and Materials

(a) *Vitamin standards.*—Ascorbic acid, Niacinamide, Pyridoxine Hydrochloride, Thiamine Hydrochloride, and Riboflavin (USP Reference Standards). Alternatively, in-house working standard such as thiamine mononitrate, assayed against USP Reference Standard, may be used.

(b) *Chemicals.*—Triethylamine (Matheson Coleman and Bell), 2-monothioglycerol (Evans Chemetics, Inc.), heptanesulfonic acid, sodium salt, LC grade (Fisher Scientific Co.), methanol (Burdick and Jackson Co.), and glacial acetic acid, LC grade (Baker).

(c) *Mobile phase.*—Transfer 2.20 g heptanesulfonic acid sodium salt and 100 mg EDTA to large beaker containing ca 1.5 L glass-distilled water. Add ca 48 mL glacial acetic acid and pipet 10 mL triethylamine into aqueous solution. Mix well and measure pH. If pH is not 3.6 ± 0.05, adjust with acetic acid or triethylamine. Transfer aqueous solution to 2 L glass-stopper cylinder and dilute to 1700 mL with water. Add 300 mL LC grade methanol. Filter solution through 0.45 μm Teflon filter. Degas briefly before use.

(d) *Buffer solution, pH 3.6.*—Add 48 mL glacial acetic acid and 10.0 mL triethylamine to 1.5 L water. Mix well and measure pH. If pH is not 3.6 ± 0.05, adjust with acetic acid or triethylamine. Transfer aqueous solution to 2 L glass-stopper cylinder and dilute to 1700 mL with water.

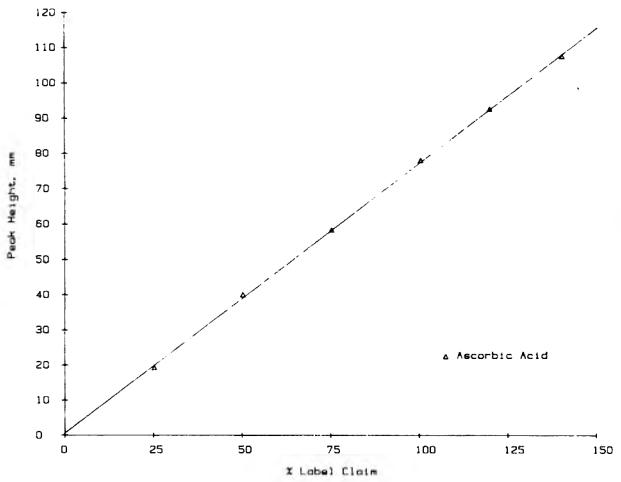

Figure 1. Linearity study of ascorbic acid at 254 nm.

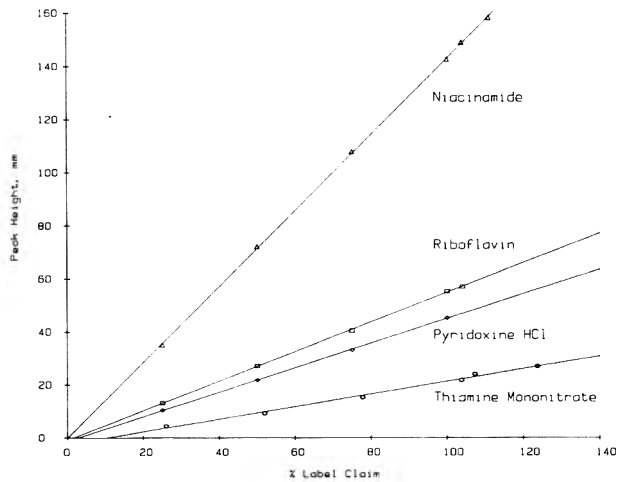

Figure 2. Linearity study of niacinamide, riboflavin, pyridoxine, and thiamine mononitrate at 280 nm.

Assay Preparations

(a) *Dosage forms without iron.*—Carefully grind 5 tablets to fine powder using glass mortar and pestle. Transfer quantitatively to 1 L volumetric flask. Add 10 mL monothioglycerol and 800 mL buffer solution. Sonicate 30 min. Add 150 mL methanol and dilute to volume with pH 3.6 buffer solution. Filter portion of solution through GF/C paper and discard first few mL. Pipet 10 mL filtrate into 25 mL low-actinic volumetric flask. Dilute to volume with mobile phase.

(b) *Dosage forms with dioctyl sodium sulfosuccinate (DDS).*—Carefully grind 5 tablets and transfer to 1 L low-actinic volumetric flask. Add 10 mL 2-monothioglycerol and 1.0 g barium chloride, and dilute to volume with pH 3.6 buffer solution. Vigorously stir mixture 30 min. Filter through GF/C paper and discard first few mL.

(c) *Dosage forms with iron.*—Determine average content weight of 20 capsules. Combine capsule contents. Mix well and transfer accurately weighed amount of sample equivalent to one capsule to 100 mL low-actinic volumetric flask. Add 5 mL 2-monothioglycerol, 2.0 mL glacial acetic acid, and 75 mL pH 3.6 buffer solution. Sonicate 5 min and dilute to volume with pH 3.6 buffer solution. Vigorously stir mixture 30 min and filter through GF/C paper into 250 mL low-actinic Erlenmeyer flask. Add 300 mg cupferron to solution. After stirring additional 10 min, let reaction mixture stand at room temperature 1 h and then filter through GF/C paper. If precipitate forms after filtration, let solution settle additional 30 min. Clear solution should be obtained after second filtration through glass fiber paper. Discard first few mL.

Apparatus

Analyses were performed using a Waters radial compression system (RCM) with an autosampler (Waters WISP 710) equipped with a high pressure pump (LDC Constametric II). The analytes were monitored by 2 detectors in series at 254 nm (LDC Spectro Monitor III) and 280 nm (Perkin Elmer LC-55). A Waters reverse phase C_{18} cartridge, 8 mm × 10 cm (Radial Pak A), and a 10 mV recorder (Linear, Model 385) were used. Peak heights or areas were integrated by Hewlett Packard Model 3354.

Procedure and Calculations

Set up liquid chromatograph and let equilibrate 30 min with mobile phase flowing at 2.0 mL/min. Set the 2 detector sensitivities at 0.02 (280 nm) and 2.0 (254 nm) AUFS, respectively. Use peak height method of quantitation. Calibration plot (Figures 1 and 2) is linear and passes through zero so that single-point standard method is used for calculations:

$$\text{Analyte, mg/tablet} = (C_r/500) \times (h_u/h_s) \times (1000/5)(25/10) = C_r \times (h_u/h_s)$$

where C_r is the total weight of individual vitamin in the standard preparation; h_u and h_s are the average peak heights of assay preparation and standard preparation, respectively.

Standard Preparation

The vitamin reference standard amounts used correspond to the weights in dosage forms.

Accurately weigh ca 20–110 mg niacinamide, 1–15 mg pyridoxine hydrochloride, 3–12 mg thiamine mononitrate (in-house standard), and 2–12 mg riboflavin, and transfer to 500 mL low-actinic volumetric flask. Add 10 mL monothioglycerol, and then add 400 mL pH 3.6 buffer solution. Sonicate 30 min. Add 75 mL methanol, mix, and dilute to volume with pH 3.6 buffer solution. Use as stock solution and keep in the refrigerator.

Accurately weigh ca 20–80 mg (⅕ unit formula weight/tablet) ascorbic acid, and transfer to 100 mL low-actinic volumetric flask. Dilute to volume with stock solution. Use as standard solution.

Results and Discussion

Early methods for the chromatographic assay of water-soluble vitamins were tedious and of limited utility (2–6) because each of the 5 vitamins (ascorbic acid, niacinamide, thiamine, riboflavin, and pyridoxine) has unique chemical characteristics, such as stability, polarity, and acidity. With the recent advance of ion-pairing chromatography (6), the effects of organic solvent, pH, and counter ion are becoming well understood. Technicon (7) and Waters Associates (8) have since reported the LC assay of 4 vitamins (niacinamide, thiamine, riboflavin, and pyridoxine) by ion-pairing technique. A more comprehensive paper about the simultaneous separation of ascorbic acid, niacinamide, pyridoxine, thiamine, and riboflavin in multivitamin preparations was reported by Jenkins (9) of Hoffman-La Roche. The success of the latter method, however, seems to be limited to vitamin formulations which do not contain minerals such as iron and magnesium.

Table 1. Effect of pH and amount of amine modifier on separation of water-soluble vitamins in multivitamin preparations

Run	Mobile phase[a]	pH	Eluting volume, mL					
			Triethylamine, %	Ascorbic acid	Niacinamide	Pyridoxine	Thiamine	Riboflavin
1	1	3.2	0.8	2.38	5.61	6.11	13.20	15.35
2	1	3.4	0.8	2.20	4.29	5.08	11.88	15.18
3	1	3.4	1.0	2.20	4.29	5.06	11.55	15.62
4	1	3.6	0.8	2.48	6.60	7.92	17.84	29.7
5	1	3.6	0.5	2.52	7.20	10.2	29.7	39.9
6	1	3.6	0.2	3.60	7.80	19.2	94.8	80.4
7	2	3.6	0.2	3.00	7.56	14.64	62.88	45.6

[a]Mobile phases: (1) Methanol–water (15 + 85) containing 0.005M heptanesulfonic acid sodium salt.
(2) Methanol–water (20 + 80) containing 0.005M heptanesulfonic acid sodium salt.

Table 2. Recovery and accuracy study of ascorbic acid (mg/unit) from spiked placebo[a]

Sample	Formula I			Formula II			Formula III			Formula IV		
	Added	Found	Rec., %	Added	Found	Rec., %	Added	Found	Rec., %	Added	Found	Rec., %
1	423.0	424.3	99.69	291.0	291.4	100.1	41.00	41.63	101.5	69.70	70.92	101.8
2	474.0	476.4	99.50	314.7	312.8	99.4	48.10	46.94	97.6	78.25	77.90	99.6
3	507.0	506.0	99.80	322.2	321.9	99.9	51.04	52.47	102.8	84.35	83.13	98.6
4	517.5	515.9	99.69	328.3	327.1	99.6	51.40	51.35	99.9	85.80	83.12	96.9
5	524.5	521.5	99.43	333.2	330.2	99.1	52.47	51.52	98.2	86.80	84.96	97.9
6	530.5	528.3	99.58	357.2	353.0	98.8	53.26	53.62	100.6	87.70	85.18	97.1
7	542.5	538.2	99.21				54.39	53.81	98.9	89.38	87.07	97.4
8	579.5	570.0	98.36				57.25	56.89	99.4	95.60	91.97	96.2
Av.			99.41			99.5			99.9			98.2
RSD, %			0.5			0.5			1.7			1.8
Conf.[b]			1.4			1.5			5.2			5.4

[a]Formula I: 7 vitamins and 1 mineral.
Formula II: 10 vitamins and 5 minerals.
Formula III: 9 vitamins and 3 minerals (large excess of Fe).
Formula IV: 9 vitamins and 4 minerals.
[b]Confidence limit (99%), 3 SD.

Table 3. Recovery and accuracy study of niacinamide (mg/unit) from spiked placebo[a]

Sample	Formula I			Formula II			Formula III			Formula V		
	Added	Found	Rec., %	Added	Found	Rec., %	Added	Found	Rec., %	Added	Found	Rec., %
1	81.7	82.2	100.6	94.0	92.6	98.5	8.40	8.43	100.3	25.15	25.14	100.0
2	92.7	92.9	100.2	101.8	100.2	98.4	9.50	9.49	99.9	28.20	27.92	99.0
3	98.4	98.2	99.8	103.4	102.5	99.1	10.20	10.20	100.0	30.10	29.54	98.7
4	100.8	99.8	99.0	105.8	106.6	100.8	10.40	10.42	100.2	31.00	31.30	101.0
5	102.0	101.7	100.6	107.3	107.3	100.0	10.50	10.59	100.9	31.50	31.53	100.1
6	103.3	103.2	100.7	113.1	109.4	97.6	10.60	10.62	100.2	31.85	32.08	98.7
7	106.1	106.7	100.6				10.85	10.83	99.8	32.50	32.23	99.2
8	111.7	112.4	100.6				11.55	11.55	100.0	34.95	34.84	99.7
Av.			100.3			99.1			100.2			99.5
RSD, %			0.6			1.2			0.3			0.9
Conf.[b]			1.8			3.6			1.0			2.7

[a]Formula I: 7 vitamins and 1 mineral.
Formula II: 10 vitamins and 5 minerals.
Formula III: 9 vitamins and 3 minerals (large excess of Fe).
Formula V: 9 vitamins and 4 minerals (with dioctyl sodium sulfosuccinate).
[b]Confidence limit (99%), 3 SD.

Table 4. Recovery and accuracy study of pyridoxine HCl (mg/unit) from spiked placebo[a]

Sample	Formula I			Formula II			Formula III			Formula V		
	Added	Found	Rec., %	Added	Found	Rec., %	Added	Found	Rec., %	Added	Found	Rec., %
1	8.68	8.75	100.8	6.87	6.96	101.3	2.52	2.51	99.6	4.40	4.39	99.8
2	9.82	9.81	99.9	7.43	7.45	100.3	2.84	2.83	99.5	4.95	4.91	99.2
3	10.71	10.73	100.2	7.53	7.57	100.5	3.06	3.05	99.7	5.34	5.20	97.4
4	10.93	10.88	99.5	7.81	7.82	100.1	3.12	3.13	100.2	5.45	5.43	99.6
5	10.98	11.02	100.4	7.83	8.00	102.1	3.13	3.17	101.2	5.50	5.49	99.8
6	11.10	11.07	99.7	8.41	8.62	102.6	3.19	3.20	100.3	5.56	5.56	100
7	11.30	11.27	99.7				3.24	3.23	99.8	5.67	5.66	99.8
8	12.16	12.13	99.8				3.46	3.46	100.0	6.05	6.02	99.5
Av.			100.0			101.2			100.0			99.4
RSD, %			0.4			1.0			0.6			0.8
Conf.[b]			1.3			3.1			1.7			2.5

[a,b]See Table 3.

Table 5. Recovery and accuracy study of thiamine mononitrate (mg/unit) from spiked placebo[a]

	Formula I			Formula II			Formula III			Formula V		
Sample	Added	Found	Rec., %	Added	Found	Rec., %	Added	Found	Rec., %	Added	Found	Rec., %
1	22.12	21.95	99.23	10.02	9.87	98.5	2.64	2.64	100.0	6.00	6.05	100.8
2	24.87	24.80	99.72	10.82	10.61	98.0	2.96	2.95	99.7	6.75	6.87	101.8
3	26.22	25.63	97.75	11.02	10.77	97.7	3.20	3.18	99.4	7.28	7.47	102.6
4	27.26	27.45	99.75	11.22	11.37	101.3	3.27	3.22	98.4	7.43	7.59	102.1
5	27.44	27.40	99.85	11.62	11.65	100.2	3.30	3.30	100.0	7.50	7.71	102.8
6	27.84	27.25	97.88	12.40	12.16	98.1	3.34	3.31	99.1	7.58	7.75	102.2
7	28.38	27.97	98.69				3.40	3.33	97.9	7.73	7.94	102.2
8	30.24	30.03	99.1				3.64	3.57	98.1	8.29	8.39	101.2
Av.			99.02			99.0			99.1			102.0
RSD, %			0.8			1.4			0.8			0.7
Conf.[b]			2.5			4.4			2.5			2.2

[a,b]See Table 3.

Table 6. Recovery and accuracy study of riboflavin (mg/unit) from spiked placebo[a]

	Formula I			Formula II			Formula III			Formula V		
Sample	Added	Found	Rec., %	Added	Found	Rec., %	Added	Found	Rec., %	Added	Found	Rec., %
1	13.33	13.29	99.7	10.34	10.25	99.1	1.73	1.70	98.3	5.29	5.26	99.2
2	14.82	14.86	100.3	11.13	10.99	98.7	1.94	1.92	99.0	5.95	5.91	99.3
3	16.10	16.10	100.0	11.34	11.31	99.7	2.06	2.07	100.5	6.41	6.25	97.5
4	16.36	16.40	100.4	11.64	11.68	99.7	2.08	2.06	99.0	6.54	6.56	100.3
5	16.56	16.71	100.9	11.84	11.76	99.3	2.12	2.12	100.0	6.61	6.57	99.4
6	16.65	16.88	101.4	12.66	12.60	99.5	2.14	2.15	99.1	6.68	6.80	101.8
7	16.97	17.00	100.2				2.17	2.15	99.1	6.81	6.77	99.4
8	18.20	18.29	100.5				2.34	2.36	100.9	7.27	7.22	99.3
Av.			100.4			99.3			99.7			99.5
RSD, %			0.5			0.4			0.9			1.2
Conf.[b]			1.6			1.2			2.8			3.3

[a,b]See Table 3.

Table 7. Stability study of the chromatographic system (time period of study: 3½ h)

	Peak height, mm				
Sample	Ascorbic acid[a]	Niacinamide[b]	Pyridoxine HCl[b]	Thiamine mononitrate[b]	Riboflavin[b]
1	99.5	146.5	71.2	22.5	56.5
2	99.0	146.0	71.2	22.5	56.2
3	99.2	146.0	71.5	22.6	56.2
4	99.0	146.8	71.5	22.8	57.0
5	99.0	146.8	71.5	22.5	57.2
6	99.2	147.2	71.5	22.5	57.2
7	99.0	147.2	71.2	22.4	57.5
8	99.5	147.8	72.0	22.4	58.0
9	99.6	148.0	72.0	22.4	58.0
10	99.5	147.5	71.8	22.5	57.8
Range	99.0–99.6	146.0–148.0	71.2–72.0	22.4–22.8	56.2–58.0
Av.	99.25	146.92	71.54	22.51	57.16
SD	0.025	0.73	0.31	0.12	0.69
RSD, %	0.25	0.50	0.43	0.53	1.21

[a]Monitored at 254 nm, 2.0 AUFS.
[b]Monitored at 280 nm, 0.02 AUFS.

Table 8. Comparison of liquid chromatographic assay with autoanalyzer assay of water-soluble vitamins in commercial multivitamin-mineral dosage forms

	Ascorbic acid, mg		Niacinamide, mg		Pyridoxine HCl, mg		Thiamine mononitrate, mg		Riboflavin, mg	
Formulation	LC	AA	LC	AA	LC	AA	LC	AA	LC	AA
I	517	518	102.2	101.6	10.66	10.85	26.82	27.31	16.34	16.50
II	308	298	104	106	7.00	7.20	10.37	10.10	10.97	11.20
III	50.5	52.0	10.6	10.9	3.30	3.19	3.18	3.20	1.96	2.10
V	—	—	29.4	28.4	4.77	4.90	6.57	6.42	6.27	6.11

Mobile Phase Effect

We studied in detail the effects of pH and amount of amine modifier in our mobile phase on separation of water-soluble vitamins in multivitamin preparations (Table 1). The amount of amine modifier in the mobile phase is extremely critical for the separation of niacinamide and pyridoxine. Decreasing triethylamine from 0.5 to 0.2% in a pH 3.6 mobile phase, significantly increases the retention times of all 5 water-soluble vitamins. Increasing the methanol/water ratio from 15 to 20%, however, elutes riboflavin faster and reverses the

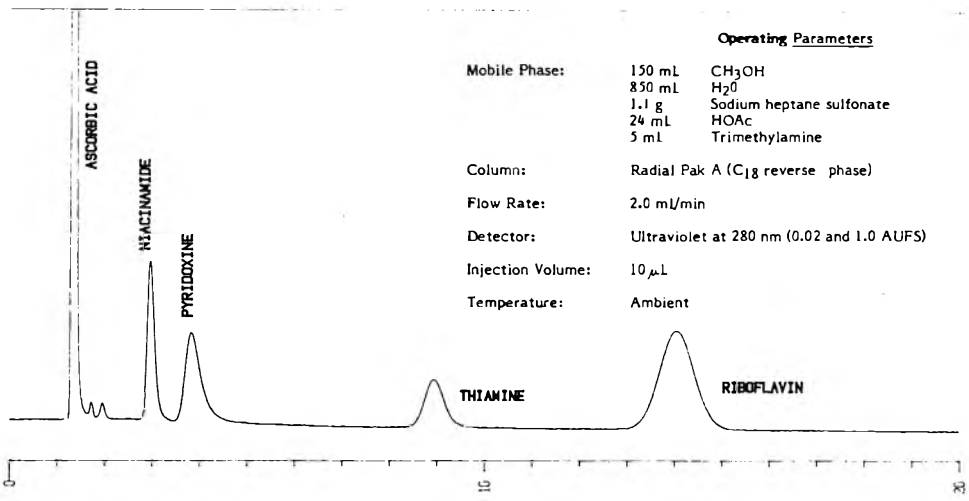

Figure 3. LC separation of 5 water-soluble vitamins.

order of separation of thiamine and riboflavin. We have concluded that a pH 3.6 mobile phase of methanol–water (15 + 85), 0.005M heptanesulfonic acid, and 0.5% triethylamine gives an optimal separation of 5 water-soluble vitamins: ascorbic acid, niacinamide, pyridoxine hydrochloride, thiamine mononitrate, and riboflavin (Figure 3).

Effect of Minerals and Dioctyl Sodium Sulfosuccinate

In our work on the LC assay of folic acid (10), we noted that the presence of minerals, magnesium oxide (MgO) in particular, interferes with its assay. Jenkins (9) also reported the interference from MgO and other minerals and was unable to quantitate the water-soluble vitamins in 3 of 8 formulations. Our sample preparation, however, removes the excess of ferrous sulfate (or ferrous fumarate) and DDS, enabling successful quantitation of the water-soluble vitamins. The large excess of ferrous fumarate or sulfate interferes with the assay of ascorbic acid. However, ferrous sulfate or fumarate can be removed by the addition of cupferron (ammonium salt of *N*-nitrosophenylhydroxylamine) to form a water-insoluble complex which is removed by filtration. We found that DSS in the formulation gives a low recovery for thiamine. (DSS was removed by the addition of a suitable amount of $BaCl_2$ followed by filtration.) Ascorbic acid cannot be assayed by our LC method in some multivitamin products. Although large quantities of $CaCO_3$ affect the peak height of pyridoxine, addition of similar amounts of $CaCO_3$ to reference standards will compensate to give excellent assay results.

Linearity Study

Concentrations at approximately 25% intervals from 25 to 150% label claim were assayed by the proposed method. Figures 1 and 2 show that peak height vs concentration plots were linear and passed through the origin. The intercepts (b) and slopes (m) have been determined by the equation $y = mx + b$. The coefficients of variation (r^2) were also calculated and ranged from 0.99 to 1.00.

Recovery and Accuracy Study

A combination study of accuracy of the method and recovery from a placebo in the range of 80–100% of unit formula of ascorbic acid, niacinamide, pyridoxine, thiamine, and riboflavin was done for a commercial multivitamin-mineral preparation. Eleven formulations of solid dosage forms (with minerals) were carefully studied and recoveries were 98.2–102% (Tables 2–6).

Stability of the Chromatographic System

The stability of the chromatographic system was determined by making 10 consecutive injections of reference standard solution over a 3½ h period. The results showed a percent relative standard deviation of ≤ 0.55 for all vitamins except riboflavin, which had a value of 1.2 (Table 7).

Commercial Multivitamin-Mineral Preparations

The method has been applied to 11 commercial preparations. Table 8 presents some of the assay results. In general, good agreement was obtained between the LC and the automated methods.

Summary

Even though most commercial vitamin formulations contain a number of different minerals, all 5 vitamins can be separated by modifying the assay preparation according to the mineral content of the formulation. For example, a large amount of iron that interferes with the assay of ascorbic acid can be removed by reaction with cupferron to form an iron complex that is insoluble in aqueous solution. Similarly, although large quantities of $CaCO_3$ affect the peak height of pyridoxine, addition to reference standards of similar $CaCO_3$ amounts will compensate to give excellent results. The addition of DSS to the formulation, which gives a low recovery of thiamine, can be overcome by removing DSS in the solution with barium chloride.

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Simultaneous Determination of Thiamine and Riboflavin in Foods by Liquid Chromatography

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Recent methods for determination of thiamine (thiochrome) and riboflavin by liquid chromatography (LC) are outlined and discussed, and a new method allowing the simultaneous determination of these 2 vitamins by using a single fluorescence detector is described. This system involves an ODS 5 μm ultrasphere column and a pH 7.5 mobile phase composed of 0.005M tetrabutyl ammonium phosphate in methanol–water (20 + 80).

The determination by liquid chromatography (LC) of water-soluble vitamins including thiamine and riboflavin, singly as well as simultaneously, has been known for over a decade. However, major breakthroughs in this area were limited mostly to analysis (or testing) of standard mixtures, pharmaceutical preparations, and vitamin concentrates (1–4). There have been few developments in the determination of vitamins in nonfortified food systems. This paper covers the history and state-of-the-art for simultaneous analysis of thiamine and riboflavin by LC.

Van De Weerdhof et al. (1) were probably the first group to successfully determine thiamine and riboflavin by LC in nonfortified food systems. They used columns of different lengths filled with 20–30 μm silica gel. The mobile phases consisted of a pH 6.8, 0.1M phosphate buffer in 10% ethanol and a pH 4.8, 0.1M acetate buffer for eluting thiamine and riboflavin, respectively. Fluorescence detection was used for both vitamins. Thiamine was oxidized to thiochrome in a post-column system. No quantitative data or information on elution time was provided. Column life was reported to be only a few days.

Toma and Tabekhia (5) described the simultaneous quantitative determination of thiamine, riboflavin, and niacin in various fortified rice products. Vitamins were separated on a 300 × 4 mm μBondapak 10 μm C18 column (Waters Associates). The mobile phase consisted of 25 mL each of PIC-5 and PIC-7, and a 600 + 390 + 10 mixture of water–methanol–acetic acid (Water Associates). Compounds were detected by UV at 254 nm. The data obtained compared well with those obtained by AACC methods (6). Recovery values, with one exception, were above 90%.

Ang and Moseley (7) failed to duplicate Toma and Tabekhia's results with meat and poultry samples because of the relatively low sensitivity associated with UV detection, and even more, because of the presence of an array of overlapping peaks, notable in cases of sample pre-injection. Instead, they developed a highly sensitive procedure for the separate determination of riboflavin as lumiflavin, and thiamine as thiochrome on a 2.1 × 500 mm 20 μm silica column, using an all-organic solvent mobile phase of chloroform–methanol (90 + 10), and fluorescence detection. The reported lower limits of detection were 0.05 and 0.2 ng for thiochrome and lumiflavin, respectively. Recovery studies yielded values ≥ 90%. No comparative study was performed involving LC and conventional AOAC methods (8). The possible simultaneous determination of the 2 compounds using a dual detection system was not explored because of detector limitations.

Kamman et al. (9), in an effort to overcome what they considered rather lengthy derivatization procedures by Ang and Moseley, developed a method for the simultaneous determination of thiamine and riboflavin, using a reverse phase ion-pairing system, UV detection, a μBondapak C18 column, and a mobile phase containing a pH 7.0, acetonitrile–aqueous 0.01N phosphate buffer (12.5 + 87.5) and 0.005M heptanesulfonic acid sodium salt. Separations were successful only with fortified food systems. The relative detection limits at 254 nm for thiamine and riboflavin were reported to be 30 and 5 ng, respectively. The results of the LC method compared favorably with those obtained from a semiautomatic modification of the AOAC method.

Skurray (10) reported the successful separation not only of thiamine (as thiochrome) and riboflavin, but of niacin as well, using a 3.9 × 300 mm 10 μm μBondapak C18 column, and an all-aqueous mobile phase composed of 0.2M acetate buffer and 0.005M heptanesulfonic acid. Thiamine and riboflavin were detected by fluorescence, and niacin by UV at 250 nm. An alkaline potassium ferricyanide solution converted thiamine to thiochrome. This highly alkaline extract apparently was injected into the column without further pH adjustment. Excellent separations were achieved with a wide variety of food matrices including bovine muscle, whole milk, yogurt, peas, herring, white bread, corn flakes, and rolled oats. The data were reported to be in good agreement with those obtained by manual AOAC methods.

Fellman et al. (11) described a method for simultaneous determination of thiamine and riboflavin in several food systems, which differs from the above methods both in sample preparation and LC separation. Using the common extract concept, thiamine was again oxidized to thiochrome. Immediately following oxidation, the extract was neutralized and then purified and concentrated on a Sep-Pak C18 cartridge (Waters Associates). This pre-injection procedure eliminated any excess potassium ferricyanide from the extract and the high sample pH, which can be detrimental to LC column life and stability of riboflavin. The two vitamins were separted on a 8 × 100 mm 10 μm Radial-Pak C8 column (Waters Associates) with a pH 7.0 mobile phase of methanol–0.01M phosphate buffer (37 + 65). The presence of interfering substances necessitated a separate wavelength system for riboflavin.

Method Development and Comparison

With a Waters Associates Model 420 fluorescence detector with excitation and emission wavelengths of 450 and 530 nm for riboflavin, the lower detection limit was 1.0 ng. With a Turner Model 111 fluorescence detector with excitation and emission wavelengths of 360 and 415 nm for thiochrome, the lower detection limits was 0.5 ng. The LC data generated in this study compared well with those obtained with the manual AOAC method for riboflavin, but were significantly lower for thiamine. The differences in thiamine values between the 2 methods were explained by failure to completely eliminate interfering substances during the purification steps with the base-exchange silica (Decalso) associated with the manual AOAC method. Modifications of the Waters fluorescence detector by replacing the instrument's dc board with an ac board and using a new internal aperture lamp (Waters Asso-

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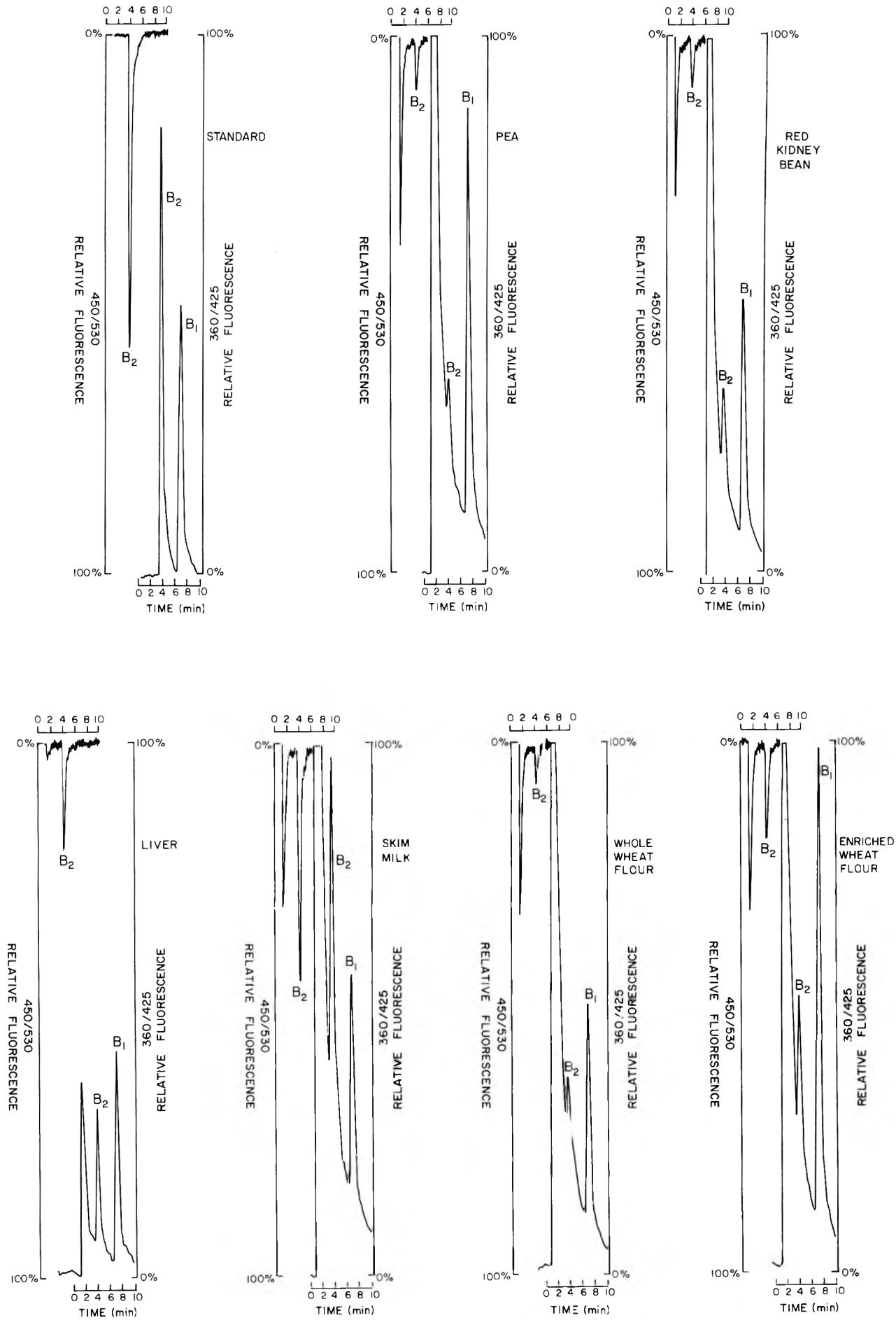

Figure 1. Chromatograms of thiamine (thiochrome) and riboflavin in standard form and in foods by using dual fluorescence detection.

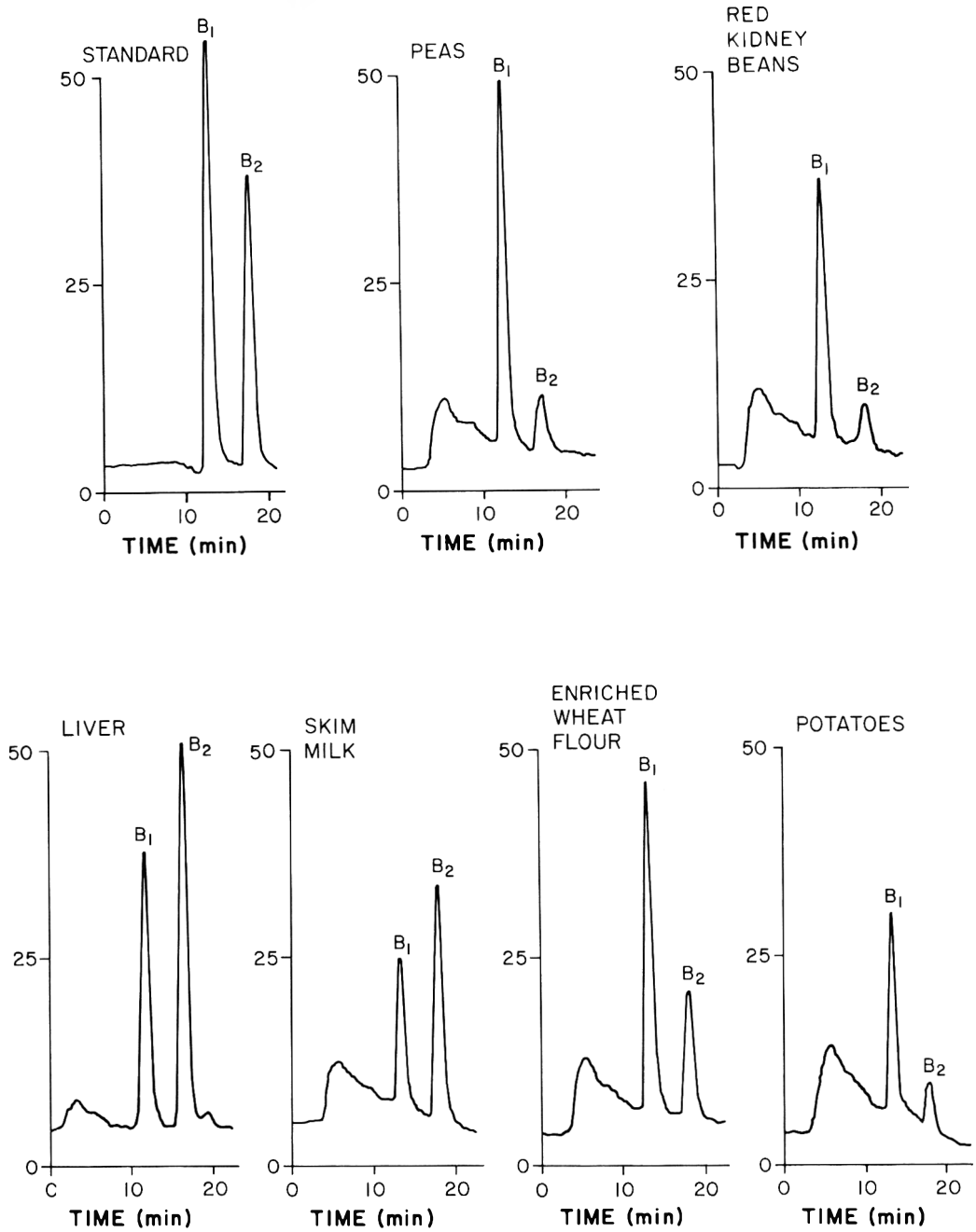

Figure 2. Chromatograms of thiamine (thiochrome) and riboflavin in standard form and in foods by using single fluorescence detection.

ciates part No. 78250) improved the lower detection limit for riboflavin to 0.1 ng. Figure 1 shows the separations obtained with this system. Using slower flow rates, in some instances, resulted in better, yet still incomplete separations of riboflavin from interfering peaks; thus, a second detector was still required. Changing to a 4.6 × 250 mm 5 μm Ultrasphere ODS column with the same mobile phase did not solve the interference problem either. An acceptable separation finally resulted with a pH 7.5 mobile phase consisting of methanol–water (20 + 80) containing 0.005M tetrabutylammonium phosphate (TBAP) (Figure 2).

Because thiochrome and riboflavin are non-ionic compounds, there is little effect with the ion-pairing TBAP. TBAP use did, however, remove the compounds which previously interfered with the separation of riboflavin (see Figure 1).

Table 1 shows the effect of methanol–water ratios in the mobile phase on the relative retention times of thiochrome and riboflavin. Interesting, but not surprising, is the short retention of thiamine which is cationic in nature and thus poorly retained by a cationic, quaternary ammonium-pairing system. An anionic system, such as hexanesulfonic acid, undoubtedly would have delayed retention time for thiamine, and thiamine would have eluted after riboflavin (5,9).

Conclusions

The LC methods have the distinct advantages over the manual AOAC method of eliminating the lengthy ion-exchange purification step and allowing the simultaneous determination of 2 vitamins. The LC procedure, however, requires the lengthy de-esterification process of thiamine phosphate esters.

Table 1. Retention of thiochrome and riboflavin in methanol–water (20 + 80) (containing 0.005M TBAP), pH 7.5

Methanol, %	Thiochrome	Riboflavin
10	45	100
15	21.5	35.5
	(4.4)[a]	
20	13.0	18.0

[a]Thiamine retention.

Kimura et al. (12) successfully separated thiochrome and its phosphate esters in standard mixtures. This system has not yet been successfully applied to food matrices, nor to the simultaneous determination of riboflavin. However, Kimura's results and the results in Table 1, which demonstrate the separating effect of decreased methanol concentration in the mobile phase on thiochrome and riboflavin, indicate a possible simultaneous separation of thiochrome and its phosphate esters as well as riboflavin. It might also be possible to separate nicotinic acid in the mixture by similar means, as described by Tyler and Shrago (13).

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Determination of Folacin in Foods and Other Biological Materials

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The objective of most methods for determination of folates in foods and other biological materials is to estimate the total folacin content of the sample. Because folacin comprises a diverse group of related compounds exhibiting similar biological activity, the analytical method must be capable of measuring all of the folates. Methods have been developed for separation of folates in their monoglutamyl form by using anion-exchange, paired-ion reverse phase, or conventional reverse phase liquid chromatography (LC). The application of these separations to determination of folates in foods and other biological materials has been limited largely by the need for development of adequate preparative methods and sufficiently sensitive and specific detection procedures. Although LC with ultraviolet absorption detection has been successful in certain limited applications, the development of fluorometric detection methods has permitted LC determination of folates in a wide range of materials. Tetrahydrofolic acid and its substituted derivatives are detected by monitoring their native fluorescence in an acid mobile phase, while folic acid and certain other folates are measured by using an oxidative post-column fluorogenic derivatization system. Methods also have been developed for determination of the polyglutamyl chain length distribution of folates in biological materials. In total, these procedures permit a direct determination and characterization of folacin compounds.

Traditionally, microbiological methods have been used to determine folacin in foods and other biological materials. Although these procedures are very sensitive, they are usually slow and may be subject to interference. Conventional quantitation of individual folate vitamers is based on slow and laborious column chromatographic separations followed by microbiological assay of collected fractions. The development and application of these procedures have been reviewed recently (1). Radiometric competitive binding assay procedures provide an alternative to microbiological methods for total folacin determination in certain products; however, accuracy of the radiometric assay is often uncertain because of nonuniform response to various folacin compounds (2).

The determination of folates in foods and other biological materials represents a difficult analytical problem because of potential existence of vitamers that have several oxidation states, several possible single-carbon substituent groups, and a polyglutamyl chain of variable length. Although determination of polyglutamyl chain length is important for some nutritional and biochemical applications, the analysis is often simplified by determination of the folates as their monoglutamyl forms after enzymatic deconjugation.

Naturally occurring folacin in most plant and animal tissues and biological fluids comprises mainly tetrahydrofolic acid (THF), 5-methyl-tetrahydrofolic acid (5-CH_3-THF), and 10-formyl-tetrahydrofolic acid (10-CHO-THF). Folic acid is probably not a significant naturally occurring form of the vitamin, but it is often found in small quantities as an oxidation product of THF in samples stored under conditions permitting exposure to oxygen. Folic acid is the form added in food fortification because of its comparative stability. Dihydrofolic acid (DHF) does not occur naturally in appreciable quantities but it may be detected as a minor oxidation product of THF.

The purpose of this article is to review instrumental separation procedures for folacin compounds, with emphasis on applications to the analysis of foods and other plant and animal tissues. Because of the importance of adequate preparative methods to the success of the analysis, major considerations in sample extraction, enzymatic treatment, and extract purification also will be discussed.

Preparative Methodology

Ordinarily, samples are extracted by homogenization in a neutral or mildly acidic buffered solution followed immediately by a heat treatment to precipitate protein and release

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bound folates. Alternatively, samples may be deproteinated by using compounds such as trichloroacetic acid (3); this is avoided because of the extreme susceptibility of THF to oxidative degradation in acidic media.

A critically important aspect of all analytical methodology involving folates is the need for antioxidative compounds in all preparative phases of the analysis to prevent degradation of the labile reduced vitamers. The most common of the antioxidative compounds is ascorbic acid, although thiols such as 2-mercaptoethanol and dithiothreitol have been used in many applications. The comparative effectiveness of these compounds has been a matter of controversy and appears to depend on factors including pH and temperature (4). Recently, Horne and Wilson (5) reported that the presence of ascorbate in commonly used extraction buffer solutions at neutral pH actually induced undesired thermal degradation and interconversion of certain folates. Gregory et al. (6) evaluated extractions using 1.0% ascorbate at pH 4.9 and found that, under a nitrogen headspace, all folates exhibited good stability (≥90% retention) during a 60-min extraction period at 100°C. Mildly acidic extraction conditions increase the stability of ascorbic acid in the extraction buffer solution.

Another important consideration in the determination of naturally occurring folates is the lability of 10-CHO-THF and its facile conversion to 5-CHO-THF via the 5,10-methenyl-THF intermediate (7). Kinetic studies in conjunction with selection of suitable extraction conditions indicated that 10-CHO-THF is converted to the 5-formyl isomer during incubation at pH 4.9 at 100°C by a first-order process (approximate half-time = 9.6 min), with essentially complete conversion in 60 min. On the basis of these studies, it was possible to determine the 10-CHO-THF in biological materials entirely as the 5-formyl isomer, thus eliminating the problem of quantitating the mixture of 10-formyl and 5-formyl tetrahydrofolates, which arises during extractions with shorter heating times or using other pH values (6).

In the majority of food and biological analyses, the determination of the chain length of folate polyglutamates is not required. This greatly simplifies the analysis by permitting a determination of the folate vitamers in their monoglutamyl forms after treatment with a pteroylpolyglutamyl hydrolase (folate conjugase). Conjugase enzymes from a wide variety of plant and animal materials have been used in folacin assays. Many of these enzymes have yielded similar results when used in conjunction with *Lactobacillus casei* growth methods in which the organism responds equally well to folates in the mono-, di-, and triglutamyl forms. One of the more commonly used conjugase enzymes in the microbiological assay is that from chicken pancreas (8). This enzyme is not suitable for use with chromatographic methods because it yields a folate diglutamate product (9). Consequently, the results of several studies based on the use of chicken pancreas conjugase are ambiguous. Although the susceptibility of hog kidney conjugase to inhibition by certain naturally occurring compounds has been suggested, hog kidney conjugase preparations of high activity have been used successfully in the analysis of a wide variety of samples (6). Further research is essential concerning this integral phase of folacin assays.

Preparative chromatographic procedures for purification of sample extracts are essential to the success of many liquid chromatographic (LC) analyses using ultraviolet absorption or fluorometric detection systems. The first preparative chromatographic method used in conjunction with an LC method specified a hydrophobic resin to selectively retain and concentrate folate compounds (10). This resin was only marginally suitable for use in the purification of many food extracts, however. Strong cation-exchange resins have been employed for purification of sample extracts in several LC applications (10–12). Disadvantages of strong cation-exchange resins include the low recoveries obtained for 5-CHO-THF with this resin (12) and the ineffective removal of interfering compounds from certain biological extracts (11). Anion-exchange materials, such as DEAE (diethylaminoethyl)-cellulose or DEAE-Sephadex are highly effective for preparative chromatography of folates. DEAE-based ion-exchangers have been applied preparatively in the LC determination of folic acid in certain fortified foods (13) and in the determination of naturally occurring folates in foods and other biological materials (6). Preparative chromatography using sequential columns packed with a hydrophobic resin and an anion-exchanger has been employed before the LC determination of 5-CH_3-THF in blood samples (14).

Liquid Chromatography of Folacin Monoglutamates

The subtle differences among the folate monoglutamates in ionic character and hydrophobicity make them well suited for LC separations by reverse phase or ion-exchange methods. Ion-exchange LC separations of folates are similar in principle to those developed for conventional anion-exchange column chromatography. Several research groups have developed anion-exchange separations which use isocratic (15) or ionic-strength gradient methods (16–19). The first analytical application was reported by Clifford and Clifford (16), which dealt with LC analysis of several food extracts. This method was largely unsuccessful because of inadequate detection specificity and sensitivity, lack of sample cleanup, and uncertain identification of chromatographic peaks. Anion-exchange LC was successful in the determination of folates in blood plasma from individuals receiving large doses of folic acid (17), although the sensitivity of the ultraviolet detection method was inadequate for analysis of plasma from unsupplemented subjects. Other important applications of anion-exchange LC have been in the characterization of chemical reactions involving folacin compounds (18, 19).

Ion-pair reverse phase LC has been used widely for separation of monoglutamyl folates. Folacin compounds are separated by these methods, using a reverse phase column and a neutral mobile phase containing a cationic surfactant (e.g., tetrabutylammonium phosphate). Separations may be performed isocratically or with a methanol gradient (20–24). Ion-pair LC has been applied to the determination of folic acid in pharmaceuticals (25–28) and has been used for the separation of folates in biological materials before microbiological assay (22). In addition, the method of Horne et al. (24) has been adapted to a preparative scale for use in the purification of folates following chemical synthesis (Gregory, unpublished). Picciano et al. (29, 30) developed an innovative step-gradient method for folacin separations. In this method, folate standards or sample extracts were injected onto a reverse phase column which had been equilibrated with a neutral mobile phase containing tetrabutylammonium phosphate, which caused strong retention of the folates as ion-pairs. Much of the nonfolate material in the sample was then immediately eluted using a mobile phase without the ion-pairing agent, followed by separation and elution of the folates with the original mobile phase. The results of limited applications of this procedure suggest that additional sample purification, more specific detection methods, or both, would be required to permit more extensive use of this method for biological analysis (30, 31).

A paired-ion LC method was reported recently which permitted the direct determination of naturally occurring folates

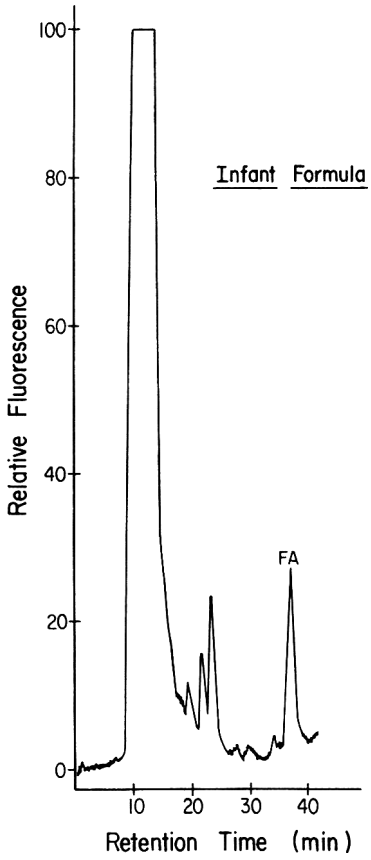

Figure 1. Reverse phase LC determination of folic acid in fortified infant formula, using post-column fluorogenic derivatization. (Reprinted from ref. 10.)

in animal tissues (12). This procedure involved sample treatment with rat liver conjugase, cation-exchange preparative chromatography, and ultraviolet absorption LC detection. A limitation of the procedure was the need to lyophilize the purified extracts to render them sufficiently concentrated for LC detection. The limited sensitivity of the procedure may limit its applicability to other samples.

Reverse phase LC using a mixed aqueous-organic mobile phase is also well suited for the separation of monoglutamyl folates. Suppression or enhancement of the ionization of folacin functional groups by controlling mobile phase pH, along with control of mobile phase polarity, are effective in achieving many separations. Day and Gregory (10) reported the first use of reverse phase LC for the separation of folacin monoglutamates. This procedure specified an isocratic acidic phosphate-acetonitrile mobile phase and coupled octadecylsilyl and phenylsilyl columns. Later studies showed that the resolution improved through the use of a 7.5–13% (v/v) acetonitrile gradient, although isocratic elution is adequate in many applications. A unique aspect of this procedure was the development of a post-column fluorogenic derivatization system. In this procedure, a hypochlorite solution was metered into the effluent stream as it exited the ultraviolet absorption detector to oxidize folic acid, DHF, and THF to highly fluorescent pterins. This greatly enhanced detection sensitivity and specificity for these compounds (10), although 5-CH_3-THF and 5-CHO-THF are resistant to oxidative cleavage and do not exhibit a significant fluorescence in this post-column derivatization system. The reverse phase method with sequential ultraviolet absorption and oxidative fluorogenic detection has been successfully applied to the determination of folic acid in fortified cereals and infant formulas (Figure 1; Table 1) (10, 11). A distinct advantage of this procedure for

Table 1. Folacin content (nmol/g) of fortified infant formula and breakfast cereal products as determined using reverse phase LC, *L. casei*, and radiometric assay procedures[a]

Procedure	Infant formula	Oat flakes
Label claim	0.25	8.0
L. casei	0.430 ± 0.007a	21.1 ± 0.8a
Radiometric	0.127 ± 0.011b	17.8 ± 2.2a
LC ($A_{280\,nm}$)	0.407 ± 0.014a	—
LC (fluor.)[b]	0.398 ± 0.004a	14.1 ± 0.6b

[a]Mean ± SD, triplicate analyses. Values in each column with the same letter were not significantly different ($P<0.05$).
[b]Fluor. refers to post-column fluorogenic derivatization method.

the analysis of fortified foods is that it has sufficient sensitivity and specificity to eliminate preparative chromatography for extract purification. In a somewhat similar method, reverse phase LC with ultraviolet absorption detection has been used for the determination of folic acid in fortified foods after sample extract purification on DEAE-Sephadex (13).

Further research was conducted to extend the applicability of the reverse phase LC procedure, particularly to the detection of other folate compounds by fluorometric methods. We found that reduced folates such as THF, 5-CH_3-THF, and 5-CHO-THF were highly fluorescent when excited at 290–295 nm in the acidic environment of the mobile phase used in the reverse phase system of Day and Gregory (pH 2.2). This observation, in conjunction with the post-column derivatization method for the fluorometric detection of folic acid, provided a means for the fluorometric determination of all folates in foods and other biological materials. When used with DEAE-Sephadex purification of the sample extract and gradient elution to improve resolution in the LC separation, this method permits the quantitation of folates in a wide range of samples (6). Figure 2 and Table 2 present typical chromatograms and quantitative results. The results of recovery, radioisotopic tracer, and fluorescence spectral studies support the accuracy of this LC procedure (6). However, the variable correlation observed between the LC and microbiological assay results indicate the need for further studies of the factors which influence the accuracy of both assay methods. Specific areas in need of further research include the potential inhibition of hog kidney conjugase in certain food extracts (e.g., orange juice) and the possible stimulation or inhibition of response in the microbiological assay. The identification of possible oxidized folates retaining biological activity is another area that requires further research, and it may explain the discrepancy between LC and microbiological assay results observed with whole wheat flour (Table 2). For samples which do not contain significant concentrations of folic acid, the rather cumbersome post-column derivatization system can be omitted to simplify the analysis.

Laukelma et al. devised an alternative method for the determination of 5-CH_3-THF in blood plasma and spinal fluid (32). This procedure involved on-column sample concentration and purification, and electrochemical detection. Methods were reported for reverse phase or anion-exchange LC, or LC on coupled reverse phase and anion-exchange columns. Although the detector response to folic acid and 5-CHO-THF was reported to be less than 0.1% of that observed for 5-CH_3-THF, the sensitivity and specificity of this detection method warrant further research into application of electrochemical detection to LC of folacin.

Liquid Chromatography of Folacin Polyglutamates

The highly ionogenic nature of the folacin polyglutamates makes them well suited for separations by anion-exchange

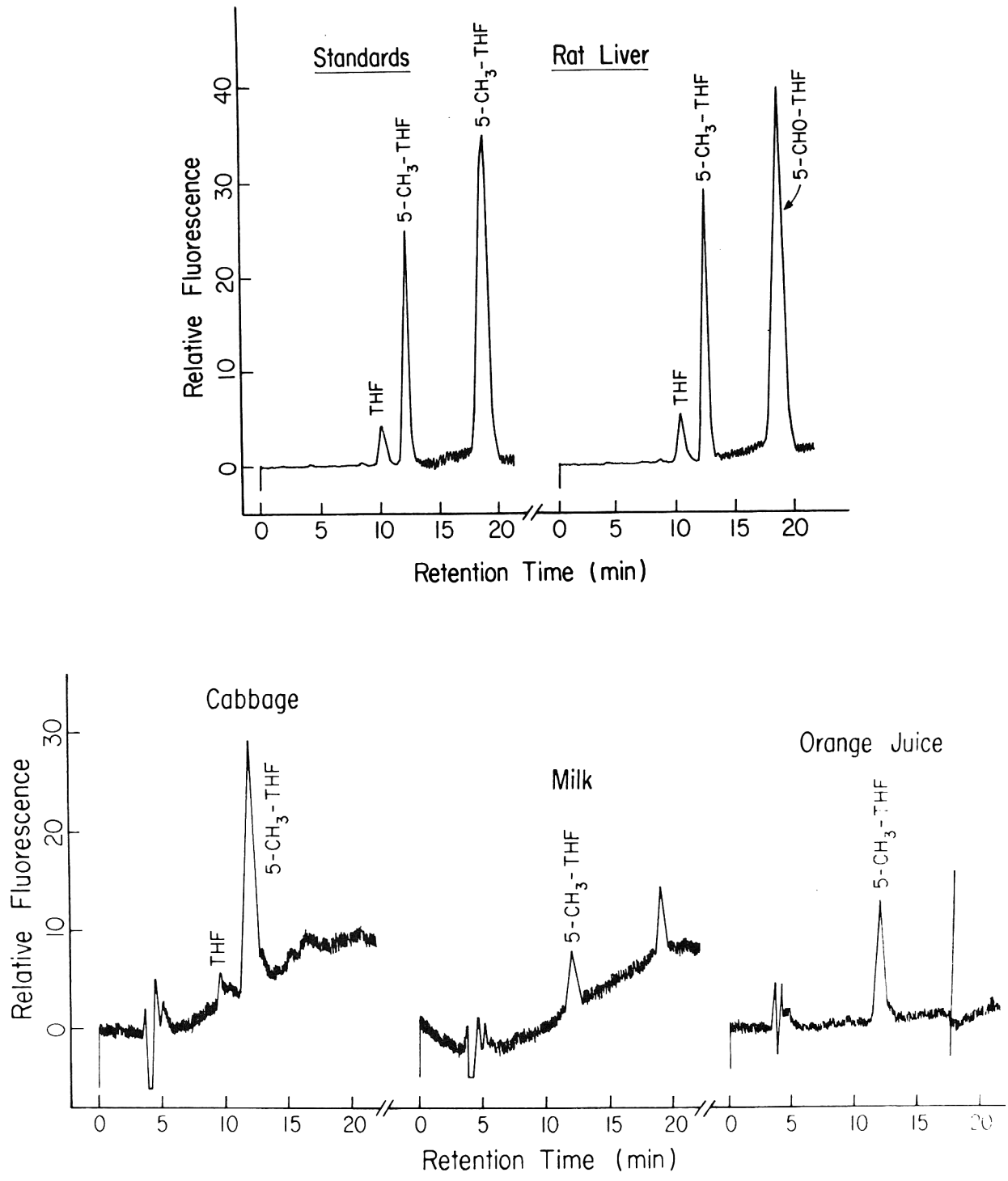

Figure 2. Determination of naturally occurring folates by reverse phase LC with direct fluorometric detection. Top chromatograms are reduced folate standards and a rat liver extract; bottom chromatograms are extracts from selected food samples. (Reprinted from ref. 6.)

LC (33, 34) and reverse phase LC (34, 35). Each of the folate compounds may exist naturally as various polyglutamyl forms, so it is unlikely that any analytical procedure could provide a simultaneous determination of such a large array of possible folate compounds. An alternative approach is to convert the folate polyglutamates in the sample extract to a common chemical form differing only in polyglutamyl chain length, which may be separated fairly easily by LC. Two research groups have independently developed such procedures.

Eto and Krumdieck (36–38) devised a series of chemical methods for the treatment of sample extracts to convert folate polyglutamates to azo dye derivatives of their respective *p*-aminobenzoylpolyglutamate cleavage products. These compounds are then separated and quantitated by reverse phase LC using visible absorption detection, which provides adequate sensitivity for determination of endogenous folates in animal tissues (38). Selective cleavage methods were developed such that the folate polyglutamates could be identified (pool 1 = THF, DHF, and 5,10-methylene-THF; pool 2 = 5-CH_3-THF; and pool 3 = 5,10-methenyl-THF, 5-CHO-THF, 10-CHO-THF, and 5-formimino-THF). Use of a radiolabeled azo dye for derivatization would permit determination of the folate polyglutamate distribution in materials with very low folate concentration (39). A possible limitation of this approach

Table 2. Folacin content (nmol/g) of selected foods and other biological materials[a]

Sample	Direct analysis						Publ. value[b]
	THF	5-CH_3-THF	CHO-THF	Folic acid	Total	*L. casei*	
Whole milk[c]	ND	0.072	ND	ND	0.072 ± 0.005	0.027 ± 0.005	0.11
Cabbage[c]	0.070	0.112	ND	ND	0.182 ± 0.008	0.293 ± 0.071	1.5
Frozen calf liver[c]	48.1	3.51	1.91	0.76	54.3 ± 4.9	36.3 ± 10.9	5.0
Frozen orange juice[c] (sample 1)	ND	0.370	ND	ND	0.370 ± 0.026	1.90 ± 0.73	1.2
Whole wheat flour[c]	ND	ND	ND	ND	ND	1.59 ± 0.31	1.2
Fresh rat liver[c]	9.64	5.51	7.10	ND	22.3 ± 0.31	NA	—
Frozen broccoli	0.690	0.370	ND	ND	1.06 ± 0.25	NA	1.3
Frozen orange juice (sample 2)	ND	0.151	ND	ND	0.151 ± 0.032	NA	1.2
Fresh orange juice	ND	0.162	ND	ND	0.162 ± 0.024	NA	1.2

[a]Mean ± SD, $n = 3$. NA = not analyzed, ND = not detected.
[b]From Perloff, B. P., & Butrum, R. R. (1977) *J. Am. Diet. Assoc.* **70,** 161–171.
[c]From Gregory et al., 1983 (ref. 6).

is the fact that 3 injections are required for each sample, and precision may be influenced by the differential calculation method.

Shane (40) devised similar cleavage methods, then analyzed with resulting *p*-aminobenzoylpolyglutamates by anion-exchange LC with ultraviolet absorption detection. Sensitivity of this procedure was sufficient for determination of endogenous folates in animal tissues and microorganisms.

Summary and Conclusions

The methods discussed in this review permit quantitative and qualitative analysis of folates. The fluorometric procedures are suitable for the LC analysis of folates as their monoglutamyl forms in a wide variety of foods and other biological materials. Recent LC methods for determination of folate polyglutamates are capable of providing valuable information for biochemical and nutritional studies. Development and application of additional methods of folacin determination will depend on suitable preparative techniques and LC detection procedures.

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TECHNICAL COMMUNICATIONS

Some Effects of Replacement of Metaphosphoric Acid/Acetic Acid Solvent System with Trichloroacetic Acid in Microfluorometric Determination of Vitamin C

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Trichloroacetic acid (TCA) is suggested as a substitute for the metaphosphoric acid/acetic acid (HPO_3–HOAc) solvent system in the AOAC microfluorometric determination of vitamin C. Comparative advantages of TCA are easier handling, greater stability, and the approximate 10-fold increase in sensitivity. For both solvent systems, the influence of acid concentration on fluorescence is described. TCA provides maximum fluorescence enhancement at pH 5.5–6.0.

Numerous methods have described the quantitative determination of vitamin C. These include titration, spectroscopic quantitation of derivatives, enzymatic and chromatographic methods, and fluorometry. All involve an initial acid extraction procedure in which a variety of acids or acid combinations are used, viz., metaphosphoric acid (1–5), acetic acid (1, 3, 6) trichloroacetic acid (7–9), perchloric acid (9, 10), oxalic acid (7), and citric acid (11, 12).

The microfluorometric method for determining both ascorbic and dehydroascorbic acids depends on the fluorescence resulting from the coupling reaction of the 1,2-dicarbonyl moiety of the sample with *o*-phenylenediamine (OPDA) in a suitable acidic solvent system. The chemistry of such quinoxaline formation from 1,2-dicarbonyl compounds and OPDA is well known (13), and several investigations into this reaction have been described (14–19). The structural identity of the fluorescent product(s) from the coupling reaction of dehydroascorbic acid and OPDA has yet to be unequivocally determined. At least 2 different quinoxaline structures have already been given (1, 20).

Utilization of the fluorescence of quinoxalines for a well described quantitative determination of vitamin C has been published by Deutsch and Weeks (1). As a result of their work, AOAC, in 1980, adopted a standard microfluorometric determination of both ascorbic and dehydroascorbic acids in which a mixture of metaphosphoric and acetic acids (HPO_3–HOAc) was used as the acidic solvent system (21). This method was used to measure total vitamin C (22) in a variety of fresh fruits and vegetables (23) as part of our work on the compilation of New Zealand food composition tables. However, TCA replaced the HPO_3–HOAc solvent system, because of its easier handling, its excellent extracting and deproteinizing properties, and its higher stability in solution than HPO_3. In this method, vitamin C is determined as dehydroascorbic acid after deliberate oxidation. Therefore, it was unnecessary to use the protective properties of HPO_3 against such oxidation, which was the main reason this acid had been selected for determining vitamin C in its reduced state (3). This paper describes the advantages of this change as well as the results of our investigations into the influence of both acid systems on fluorescence.

Experimental

Reagents

All reagents were analytical grade (BDH unless otherwise noted).

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(a) *Metaphosphoric and glacial acetic acids.*—Mix and dissolve exactly as described in the AOAC method (21).

(b) *Trichloroacetic acid.*—(Riedel-de Haen and Baker). Prepare 20% (w/v) and 5% (w/v) aqueous solutions.

(c) L-*Ascorbic acid.*—Dissolve in either TCA or HPO_3–HOAc just before analysis.

(d) *Active carbon.*—Darco was used as purchased in TCA procedure, and acid-washed for AOAC method.

(e) *Boric acid.*—3% (w/v) aqueous solution.

(f) *Sodium acetate.*—(Ajax Univar.) 50% (w/v) solution of $NaOAc.3H_2O$ in water.

(g) *o-Phenylenediamine.*—0.02% (w/v) aqueous solution. Prepare fresh in the dark and use within 30 min.

Apparatus

Fluorescence was measured with a ratio fluorometer from Farrand Optical Co. Inc., New York, NY. Filters (G.K. Turner Associates, Palo Alto, CA): 7–60, excitation; 47B and 2A, emission.

Procedure

All assays using the HPO_3–HOAc solvent system were carried out exactly as described in the AOAC method. Throughout this investigation, the HPO_3–HOAc ratio was kept constant at the recommended value of 1/2.8 (w/w). Assays in which HPO_3–HOAc was replaced by TCA were carried out in duplicate by the following procedure:

Add ca 1 g active carbon to ca 25 mL vitamin C solution (see below) and shake 5 min on a wrist-action shaker. Filter through Whatman No. 42 paper and add 5 mL clear filtrate to each of two 25 mL volumetric flasks. Add 5 mL sodium acetate solution to each flask and then 5 mL boric acid solution to the blank and 5 mL water to the test solution, respectively. Shake, and after 30 min add 5 mL OPDA solution to each flask, dilute to volume with water, shake, and place in the dark. After 30 min, zero fluorometer with blank solution and measure fluorescence of test solution.

For food analyses, weigh a small amount ($\leq$ 15 g) of homogenized and, if necessary, diluted sample slurry into 25 mL 20% TCA immediately after homogenization. Stir and quantitatively transfer mixture to 100 mL volumetric flask and shake 10 min on wrist-action shaker. Dilute to volume with distilled water, mix, and filter through Whatman No. 42 paper. Use ca 25 mL clear filtrate for oxidation with active carbon and proceed as above. For external standards, dissolve an appropriate amount of vitamin C in 5% TCA and dilute with the same solvent.

For spiking, add constant amounts (V_s mL) of aqueous vitamin C solution (C_s ppm) to TCA extracts of various amounts (W_i g) of homogenized and, if necessary, diluted (dilution factor D) sample slurries. Shake, dilute to 100 mL with water and proceed as above. Plot readings against W_i and calculate slope (S) and intercept (I).

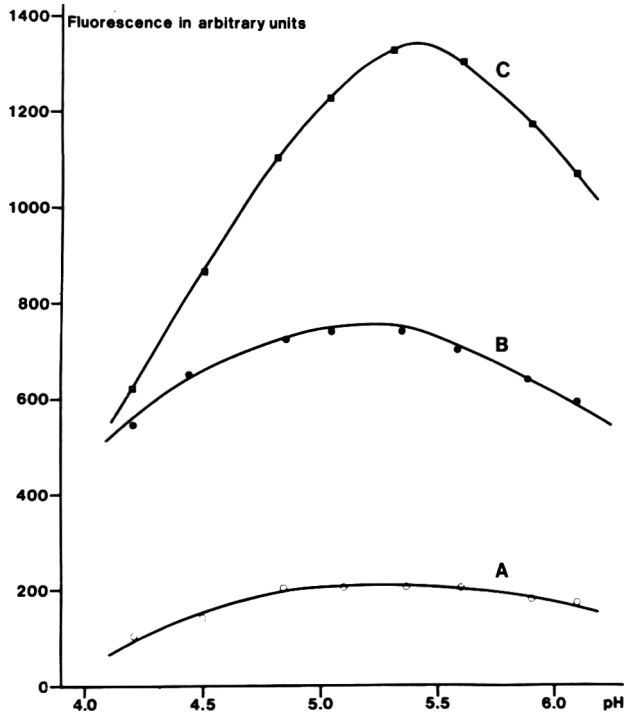

Figure 1. Fluorescence as function of pH for 3 different vitamin C concentrations, using HPO_3–HOAc solvent. Curves A, B, and C represent 30, 100, and 170 ppm vitamin C, respectively, in the sample solution.

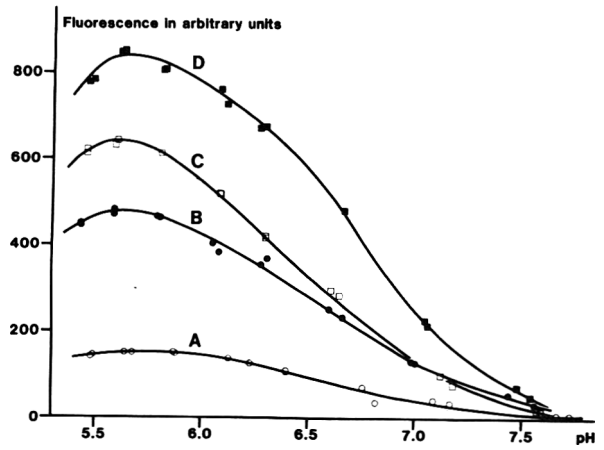

Figure 2. Fluorescence as a function of pH for 4 different vitamin C concentrations, using TCA solvent. Curves A, B, C, and D, represent 1.63, 5.34, 8.28, and 11.36 ppm vitamin C, respectively, in the sample solution.

Calculate vitamin C concentration as follows:

$$\text{mg vitamin C/100 g sample} = (S \times V_s \times C_s \times D)/(10 \times I)$$

To compare effects of TCA and HPO_3–HOAc concentrations on level of fluorescence, sets of 8 vitamin C solutions were analyzed. Within one set, vitamin C concentration was kept constant and acid concentration of the sample solutions was varied from 0.2 to 10% (w/v) in experiments with TCA and from 0.3 to 15% (w/v) HPO_3 in experiments using the AOAC method. Within one set of 8 analyses, all solutions were made from one stock solution of vitamin C by appropriate dilutions to ensure constant vitamin C concentration. Only the vitamin C solution with the highest HPO_3–HOAc concentration had to be prepared separately for practical reasons.

Three sets of vitamin C sample solutions (30, 100, and 170 ppm) were analyzed by using the method with HPO_3–HOAc. Four sets of vitamin C sample solutions of 1.63, 5.34, 8.28, and 11.36 ppm were analyzed by the TCA procedure. After the fluorescence was read, the pH of each assay solution was measured and the fluorescence reading was plotted against pH (Figures 1 and 2).

Vitamin C concentrations in samples analyzed using the HPO_3–HOAc acid systems were much greater than those using TCA to compensate for different dilutions during the actual assay in both procedures: 5 times for TCA and 70 times for HPO_3–HOAc.

Results and Discussion

Practical Aspects

The practical consequences of a change to the use of TCA in the AOAC method instead of HPO_3–HOAc can be summarized as follows: *(1)* Handling the solid acids was very different. Crystalline TCA was readily soluble. In contrast, sticks of HPO_3 needed to be crushed in a mortar in a moisture-free atmosphere before the acid could be dissolved in HOAc at a moderate rate.

(2) Dissolving HPO_3 in HOAc and then diluting with water was a time-consuming process, resulting in a slightly turbid solution. This had to be filtered through Whatman No. 2 paper to obtain a totally clear solution, which involved long filtration times. Number 4 paper improved filtration times; however, this still yielded a slightly hazy solution. Such solutions of HPO_3 were unstable and could not be kept for more than 7–10 days (3). In comparison, 20% TCA stock solutions were easy to prepare, and they could be rapidly filtered, if necessary, through Whatman No. 4 paper to remove any solid particles. The solution itself was always clear, and the stability was such that no interference with the vitamin C analysis was observed. Stock solutions of TCA were kept in the dark at room temperature. Under these conditions they were stable for at least 2 months after preparation.

(3) As a result of different dilutions in both assays proceeding from sample to assay solution (5 times for the TCA procedure and 70 times for the HPO_3–HOAc method), the sensitivity of the method was increased. However, fluorescence for a given vitamin C concentration was slightly greater for the latter procedure, resulting in a net decrease of the lower detection limit by about 10 times.

Fluorescence vs Acid Concentration

Figures 1 and 2 show that the pH level has a marked effect on the fluorescence. When HPO_3–HOAc is used as the solvent system (Figure 1), maximum fluorescence is observed at pH 5.3–5.4. For TCA (Figure 2), maximum fluorescence is observed at pH 5.6–5.7. The figures also clearly show the necessity of working at a well defined acid concentration, which was much more important than conducting the analyses at the pH of the maximum reading. For the TCA system, the pH of the maximum reading would be at about 7.5% TCA in the sample solution. The 5% TCA level at which all food samples were analyzed gave a pH of 5.8. Although this was just above the maximum value, it did not noticeably affect either the sensitivity or the accuracy. The linearity of the relationship between fluorescence and vitamin C concentration was good and application of this procedure to the analysis of over 150 samples of 36 different varieties of fruit and vegetables resulted in 96% of all duplicate analyses differing less than 4% from their respective means. Vitamin C levels were between 1.87 and 145 mg/100 g, and recoveries for each of the 36 varieties were between 80 and 128% (23).

Conclusions

There is a considerable advantage in replacing the HPO_3–HOAc solvent system by TCA in the microfluorometric determination of vitamin C. Although both procedures showed a marked influence of acid concentration on fluorescence, the consequences of this effect were easily overcome by maintaining the acid concentration at a constant value. The best results were obtained when working between pH 5.5 and 6.0 in the assay solution.

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Rapid Determination of Color Additives, Using the C_{18} Cartridge

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A reliable method has been developed for the rapid separation and identification of the 7 permitted FD&C dyes (Red Nos. 3 and 40; Blue Nos. 1 and 2; Yellow Nos. 5 and 6; Green No. 3) and the recently banned FD&C Red No. 2 in foods. The colors are separated by using the C_{18} cartridge, and their identity is confirmed by spectrophotometry.

The present methodology used for the separation and identification of synthetic colors in foods and drugs (1) is a long, tedious process invo ving 2 lengthy column chromatographic steps with Celite and cellulose. We describe a method based on reverse phase chromatography, using C_{18} cartridges for the separation of these colors.

The C_{18} cartridge (2) employs the principles of liquid chromatography to isolate, clean up, and concentrate sample components for final determination by visible spectrophotometry (3), thin layer chromatography (TLC) (4), or liquid chromatography (5). It accomplishes in a single step what usually requires a sequence of several time-consuming operations.

We report our results for the rapid determination of color additives in candy, beverage syrup, gum balls, pudding and pie filling mix, and cologne.

Experimental

Apparatus and Reagents

(a) *C_{18} cartridges* —Sep-Pak (Waters Associates, Inc., Milford, MA).

(b) *Syringe.*—10 mL with Luer tip.

(c) *Ultraviolet-visible (UV-Vis) spectrophotometer.*—Cary 118, or equivalent.

(d) *Isopropanol solutions.*—2.5, 5, 13, 20, and 50% in water.

(e) *FD&C reference standards.*—Division of Color Technology, Food and Drug Administration, Washington, DC. *Stock solution.*—100 mg/100 mL water. *Working solution.*—Dilute 1 mL stock solution to 100 mL with appropriate isopropanol solution to yield suitable spectra in 1 cm cells.

Samples

For ease of sample preparation, liquids or easily liquefied products are recommended for analysis. Acidic foods, i.e., beverages and beverage syrups, require no special preparation. Gelatin products should be dissolved in water (0.1 g in 3 mL), acidified (1 drop of 1% acetic acid), and filtered before analysis. Alcohol-containing materials (cologne) should be diluted 1:1 with water and acidified (1 drop of 1% acetic acid) before analysis to enhance dye retention (4).

Procedure

Remove plunger from 10 mL syringe, place long end of cartridge on Luer tip of syringe barrel, and pour 3 mL isopropanol into syringe barrel. Replace plunger, pump solution through cartridge, remove cartridge and discard eluate. Repeat, using 5 mL 1% acetic acid, followed by 2–3 mL filtered sample, and appropriate isopropanol solution according to scheme in Figure 1, collecting only colored portion. These steps may be repeated in succession when separating a mixture of colors requiring more than one isopropanol solution.

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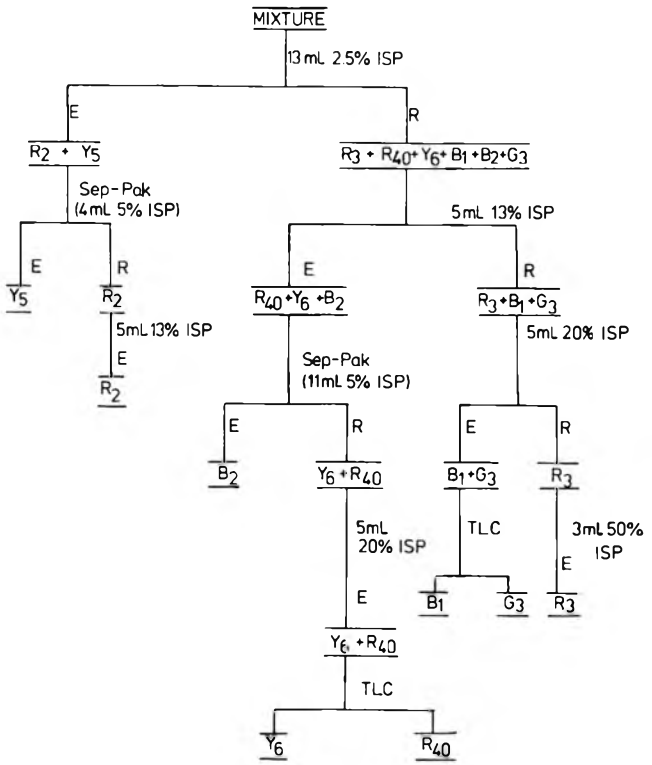

Figure 1. Color mixture schematic. Isopropanol (ISP): E, elutes and R, retains. R_2, Red No. 2; Y_5, Yellow No. 5; Y_6, Yellow No. 6; R_3, Red No. 3; R_{40}, Red No. 40; B_1, Blue No. 1; B_2, Blue No. 2; G_3, Green No. 3.

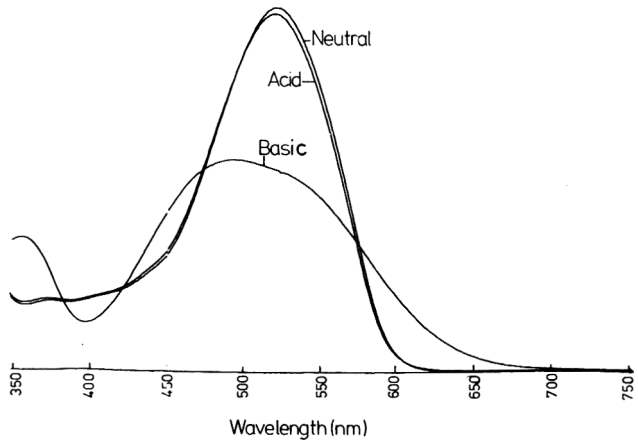

Figure 2. Spectra of FD&C Red No. 2 in 13% isopropanol.

For those color combinations requiring a second reverse phase separation (C_{18} cartridge), evaporate eluate to ca 3 mL and load onto new cartridge prepared as above. Submerge collection tubes with sample extracts in water bath. Prepare control tubes marked at 3 mL level and filled with appropriate isopropanol solution that duplicates sample volume. Heat to boiling until desired concentration level is reached.

Confirm identity of separated colors by UV-Vis spectrophotometry, using combination of neutral-acid-basic spectra (Figure 2).

Separate FD&C Yellow No. 6/Red No. 40 and FD&C Blue No. 1/Green No. 3 by TLC (6), using silica gel G plates and *n*-butanol–methyl ethyl ketone–ammonium hydroxide–water (5 + 3 + 1 + 1). Streak 3 cm band of mixture of interest along base of TLC plate and dry. Spot appropriate reference standards at spaced intervals over dried sample streak, dry, and develop ca 10 cm. One common band develops at R_f of corresponding reference standard. Depending on colors in sample, it is usually unnecessary to follow entire scheme.

Ten commercial products (cologne, pudding mix, 2 gums, 4 candies, 2 syrups) (Table 1) were analyzed by using the technique described above. To extract the colorings, the syrups, gums, and candies were diluted, washed, or dissolved in water. The cologne was evaporated to near dryness before addition of water. All solutions were filtered through paper (Whatman 2V folded) and 2–3 mL was transferred to the cartridges. The eluates were transferred to suitable spectrophotometric cells and scans were obtained from 750 to 350 nm. All UV-Vis spectra that were obtained matched standard reference spectra.

Results and Discussion

The current method (1) used for the separation of the FD&C colors in foods requires several time-consuming steps. For products containing FD&C Red No. 3 and other colors, Procedure III (1) may require 4–5 h. The major time-consuming step is the separation of the colors on a Solka-Floc column. By comparison, the procedure based on reverse phase C_{18} cartridge chromatography takes a maximum of 1 h. In many cases, when it becomes obvious that only one color is present, the procedure may require only 15 min.

The elution scheme was developed using 10 ppm concentrations of each of the 8 FD&C color additives in water. A sequence of solvent mixtures was used to selectively retain or elute the various color additives. Each successive solvent change represented a change in solvent strength beginning with the weaker solvent used to load the sample onto the cartridge. These different solutions were chosen so that particular colors in the sample would either elute rapidly with 2–7 mL solvent (e.g., FD&C Yellow No. 5, FD&C Green No. 3), or be strongly retained by the packing (e.g., FD&C Red No. 3). FD&C Red No. 2 is the only red color examined that eluted with 2.5% isopropanol, which accounts for the selection of that concentration. Sugars and flavorings are not retained by the cartridge but are flushed through upon addition of the dissolved sample.

The elution patterns may be affected by some product excipients, but if no interferences are present all analyses proceed as outlined. In lilac candy pops the FD&C Blue No. 2 eluted in 20% isopropanol instead of 13% isopropanol. We do not have an explanation for this observation. When this problem is encountered, however, the eluate is collected, corresponding reference standards are prepared in the same strength isopropanol solution, and UV-Vis spectra are obtained.

A tally was made of the most common FD&C single colors and color combinations found during routine sample analyses over a 2-year period. The single FD&C colors in descending order of occurrence were: Red No. 40, Red No. 3, Yellow No. 5, Red No. 2, Yellow No. 6, and Blue No. 1. FD&C Green No. 3 and Blue No. 2 were not found. The 5 most common FD&C color combinations were: *(1)* Yellow Nos. 5 and 6; *(2)* Yellow No. 5 and Blue No. 1; *(3)* Yellow Nos. 5 and 6, Blue No. 1, and Red No. 3; *(4)* Red No. 3 and Blue No. 1; or Blue No. 1, Yellow Nos. 5 and 6, and Red No. 40; and *(5)* Red No. 40 and Blue No. 1.

This tally was based on the number of times these colors appeared in 203 samples of products analyzed. All of the 7 permitted FD&C colors and the recently banned FD&C Red No. 2 are not likely to be found in combination at any one time. Therefore, the color mixture schematic provides a useful sequence of analysis for screening the colors.

The results of a number of color determinations are presented in Table 1. These data demonstrate the generality of

Table 1. Determination of colors in various products

Product	Product color	Isopropanol, %	FD&C colors detected
Rose syrup	red	2.5	Red No. 2
Rose syrup	red	2.5/13	Red No. 40
Gum balls	green	5	Yellow No. 5
		20	Blue No. 1
Gum balls	red-orange	13	Yellow No. 6
		50	Red No. 3
Candy pops (assorted)	lilac	20	Blue No. 2
		50	Red No. 3
	ye low-pink	5	Yellow No. 5
		13/50	Red No. 3
Strawberry milkshake candy	red & white	2.5/13	Red No. 3
		50	
Fish jelly candy	ye low	2.5/5	Yellow No. 5
Mice jelly candy	orange	2.5/5/13	Yellow No. 6
Pudding mix	white	2.5/13	Red No. 40
Florida water (cologne)	blue-green	13/50	unidentified yellow Blue No. 3

the method as a significant time-saving device in color analysis. With this system, FD&C Red Nos. 3 and 40 are easily separated. Three products, rose syrup (containing Red No. 2), pudding mix (containing Red No. 40), and strawberry milkshake candy (containing Red No. 3), demonstrate the different elution patterns of the 3 red colors: 2.5% isopropanol for Red No. 2, 13% isopropanol for Red No. 40, and 50% isopropanol for Red No. 3.

This method has been applied successfully to a variety of products and is being routinely used to rapidly screen many products received for color additive analysis in the New York Regional Laboratory.

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Quantitative Amino Acid Analysis of Feedstuff Hydrolysates by Reverse Phase Liquid Chromatography and Conventional Ion-Exchange Chromatography

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Corn, soybean meal, and isolated soybean protein samples were acid-hydrolyzed and analyzed for amino acid content by reverse phase liquid chromatography (LC) and by conventional ion-exchange chromatography (IEC) using an amino acid analyzer. The former method employed pre-column derivatization with orthophthalaldehyde (OPTA)/ethanethiol and fluorescence detection. In the LC procedure, glycine and threonine were not resolved, and proline and cyst(e)ine were not detected. In general, amino acid values obtained by LC and IEC compared closely within and across feedstuffs, and both agreed well with published amino acid composition data. The notable exceptions were aspartic acid, glutamic acid, and alanine. Results of this study suggest that reverse phase LC with pre-column OPTA derivatization can be applied to accurately measure primary amino acids in individual feedstuffs.

With regard to protein nutriture, a well-balanced diet must supply all of the indispensable amino acids in the appropriate amounts required by an organism. Therefore, to accurately formulate rations for either experimental or commercial use, the amino acid content of individual feedstuffs should be determined. Hydrochloric acid hydrolysis followed by ion-exchange chromatography (IEC) with post-column ninhydrin derivatization has been the procedure most generally followed for this purpose. More recently, liquid chromatographic (LC) methods have been developed and are gaining popularity as alternative techniques for the analysis of amino acids. Reverse phase LC procedures using pre-column derivatization with orthophthalaldehyde (OPTA) have emerged as viable alternatives for the analysis of primary amino acids in physiological fluids such as blood serum/plasma, urine, and cerebrospinal fluid (1–8) or purified protein/peptide hydrolysates (7, 9–12). However, to the author's knowledge, only one report (13) has appeared describing use of this technique for the quantitative amino acid analysis of food protein hydrolysates. In addition, one other publication (14) has appeared describing the quantitative determination of amino acids in foodstuffs by a reverse phase LC method using dansyl derivatization.

The present study was conducted to quantitatively compare a recently developed reverse phase LC technique with a conventional IEC procedure for the amino acid analysis of corn, soybean meal, isolated soybean protein, and 3 common feedstuffs of low, intermediate, and high protein content, respectively. Because the former 2 feedstuffs currently comprise the main ingredients for the majority of poultry and swine diets in the United States, a rapid, sensitive, and less expensive alternative to IEC procedures conducted via amino acid analyzers would be of interest to feed manufacturers and nutritionists.

Experimental

Reagents and Apparatus

(a) *Liquid chromatograph.*—Waters Associates (Milford, MA) LC system, previously described (8).

(b) *Amino acid analyzer.*—Beckman (Palo Alto, CA) 119 CL amino acid analyzer equipped with Model 126 data reduction system. System used cation-exchange resin (type W3),

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post-column derivatization with ninhydrin, and dual-channel absorbance detection (440 nm, 570 nm).

(c) *Chemicals.*—LC grade acetonitrile and methanol (Burdick & Jackson Laboratories, Inc., Muskegon, MI). Hydrolysate amino acid calibration standard solution (Cat. No. 312220, Beckman Instruments, Inc., Palo Alto, CA). OPTA (Fluoropa, Cat. No. 26010) and ninhydrin (Pierce Chemical Co., Rockford, IL). Ethylene glycol monomethyl ether (Fisher Scientific Co., Fair Lawn, NJ).

(d) *Buffers.*—LC buffer composition, previously described (8). Sodium citrate buffers for IEC (Beckman Instruments Co.).

Standard Preparation

(a) *LC.*—An aliquot of calibration standard solution was diluted with 99 volumes of methanol before derivatization (8).

(b) *IEC.*—An aliquot of calibration standard solution was diluted with 9 volumes of pH 2.2, 0.2N sodium citrate buffer before injection.

Sample Preparation

Corn and soybean meal (SBM) samples were each finely ground in a Burr mill (Laboratory Construction Co., Kansas City, MO) before weighing. Isolated soybean protein (ISP, Purina Assay Protein RP-100, Ralston Purina Co., St. Louis, MO) was used without further preparation. Approximately 600 mg (corn, SBM) or 300 mg (ISP) samples (one of each feedstuff) were weighed and placed into 500 mL distilling flasks. Two hundred to 250 mL 6N HCl was added, flasks were placed in a reflux apparatus, and nitrogen was bubbled into the solutions as described by Cavins et al. (15). Following 24 h reflux, hydrolysates were filtered, cooled, and diluted to 250 mL with water. One hundred mL of each hydrolysate solution was evaporated to near dryness in a rotary evaporator (60°C). Several mL water was added, and the sample was evaporated to dryness. Following a second water addition and evaporation, a concentrated hydrolysate solution (CHS) was obtained by dissolving the residue in 4 mL LC grade methanol. For reverse phase LC analysis, each CHS required a further 100-fold dilution with methanol, and 1 mL aliquots were derivatized with OPTA/ethanethiol as previously described (8). For IEC, 0.5 mL of each CHS was evaporated to dryness, and the residue was redissolved in either 1 mL (corn) or 4 mL (SBM or ISP) pH 2.2, 0.2N sodium citrate buffer. A 100 μL aliquot of each sample was then diluted to 500 μL (corn, SBM) or 1000 μL (ISP) before injection.

Chromatographic Procedures

(a) *LC.*—Duplicate standard (40 μL), corn (25 μL), SBM (10 μL), and ISP (10 μL) aliquots were injected and chromatographed as previously described (8). Forty μL derivatized standard solution contained 200 pmol each of the 13 amino acids listed in Table 1. The actual amounts of corn, SBM, and ISP analyzed per injection were 3.0, 1.2, and 0.6 μg, respectively.

(b) *IEC.*—A 92-min single column hydrolysate analysis based on the procedure recommended in the Beckman 119 CL Application Notes was followed. Three sodium citrate buffers were used to elute the amino acids. The initial system conditions were pH 3.25, 0.2N [Na^+] buffer at 50°C. At 20 min, the column temperature was increased to 65°C, where it remained for the duration of the analysis. At 22 min, the first buffer was changed to pH 3.95, 0.4N [Na^+]. The second buffer was changed at 42 min to pH 6.4, 1.0N [Na^+]. At 75 min, the column was regenerated for 2 min and re-equilibrated for 15 min with 0.2N NaOH and pH 3.25, 0.2N [Na^+] buffer, respectively. Duplicate 100 μL aliquots of the diluted calibration standard and hydrolysate solutions were injected. One hundred μL of the diluted calibration standard solution contained 25 nmol each of the 13 amino acids listed in Table 1. The actual amounts of corn, SBM, and ISP analyzed per injection were 600, 150, and 37.5 μg, respectively.

Statistics

Student's *t*-tests (18) were performed to compare LC and IEC data within and across feedstuffs.

Results and Discussion

A comparison of mean amino acid composition values for the 3 feedstuffs as determined by reverse phase LC and conventional IEC showed that, for most amino acids, the absolute values obtained were similar for the 2 methods (Table 1). The notable exceptions were aspartic acid, glutamic acid, and alanine. The LC values for aspartic and glutamic acids were actually closer to the reported values for both 50% protein SBM (15) and ISP. It appeared that an unknown component may have co-eluted with alanine during the IEC procedure. This is based on the fact that a major unknown peak appeared between tyrosine and valine in all LC analyses, whereas no extraneous peaks were observed during IEC analyses. Because SBM and ISP alanine values were abnormally high for IEC analysis, co-elution with an unknown compound could have been responsible for this observation.

Statistical analysis of the data in Table 1 revealed that, although the amino acid absolute values obtained by LC and IEC for each feedstuff were very close, in many cases they differed significantly ($P \leq 0.05$). This could be partially explained by the excellent agreement between replicate values within a method. However, the IEC procedure was less variable than the LC method, as evidenced by generally lower coefficients of variation (CV) for many of the amino acids. The average amino acid CV values were 5.2% (3.1%, excluding aspartic acid) for the LC method and 1.6% for the IEC procedure. Nevertheless, when averaged across feedstuffs, LC values for each amino acid did not significantly differ from IEC results, with only alanine approaching significance ($P = 0.061$).

Although glycine and threonine were not resolved and proline and cyst(e)ine were not detected by this method (8), the results of the present study suggest that reverse phase LC with OPTA derivatization can be used to accurately measure primary amino acids in individual feedstuffs. However, there is difficulty in detecting lysine in complete mixed feeds (unpublished data, R. G. Elkin, Purdue University, 1983). In these instances, very little lysine was detected relative to that expected based on calculated values. The major compositional difference between individual feedstuffs and complete mixed feeds is that the latter generally contain supplemental fat, vitamins, and minerals. Although purely speculative, it is possible that the mineral fraction of complete mixed feeds may interfere with the derivatization of lysine. Problems with OPTA lysine derivatization have been described, and the low fluorescence of lysine with OPTA can reportedly be enhanced by adding the detergent Brij to the reaction mixture (9, 19, 20).

According to Pfeifer et al. (21), the future of amino acid analysis lies with LC. A major advantage of LC is the adaptability of instrumentation to particular sample types or newly developed methods. Since amino acid analysis capability is inherent in today's LC systems, it is apparent that the dedi-

Table 1. Amino acid composition of corn, soybean meal (SBM), and isolated soybean protein (ISP) as determined by LC and ion-exchange chromatography (IEC)

	Corn[a]			SBM[a]			ISP[a]		
Amino acid, %	LC[b]	IEC[b]	NRC[c]	LC[b]	IEC[b]	NRC[c]	LC[b]	IEC[b]	RP[d]
Aspartic acid	0.68	0.66	NA[e]	7.56	5.01	NA	13.97	9.30	10.50
Glutamic acid	2.54	1.63	NA	4.93	3.31	NA	20.99	12.27	17.70
Serine	0.43	0.45	0.40	2.44	2.26	2.89	3.90	4.08	4.50
Histidine	0.23	0.26	0.20	1.21	1.17	1.32	1.86	1.93	2.20
Alanine	0.65	0.84	NA	2.08	7.06	NA	3.39	7.18	3.60
Arginine	0.47	0.50	0.50	3.85	3.45	3.68	6.76	5.91	6.30
Tyrosine	0.40	0.40	0.45	2.28	1.63	2.01	3.60	3.04	3.50
Valine	0.44	0.42	0.52	2.42	2.15	2.72	4.18	3.55	4.30
Methionine	0.24	0.22	0.20	0.87	0.62	0.72	1.29	0.92	1.20
Isoleucine	0.31	0.33	0.37	2.33	2.03	2.57	4.21	3.76	4.20
Leucine	1.08	1.14	1.10	3.97	3.47	3.82	7.05	6.39	7.40
Phenylalanine	0.44	0.47	0.47	2.60	2.31	2.11	4.68	4.31	4.90
Lysine	0.26	0.30	0.24	2.86	2.78	3.18	5.01	4.68	5.30

[a]Crude protein contents (%) as determined by macro-Kjeldahl procedure (ref.16): corn (8.75); SBM (49.14); ISP (88.57).
[b]Mean values for duplicate analyses.
[c]Values from Table 19 of ref. 17.
[d]Values from Technical Data Sheet No. 20-1, Purina Assay Protein RP-100 (Ralston Purina Co., St. Louis, MO).
[e]NA = not available.

cation of expensive instrumentation to only one type of analysis is neither necessary nor practical.

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Forage Nitrate Analysis: Laboratory Performance Study

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Four forage samples were analyzed by 16 laboratories for KNO_3 content. Individual reported KNO_3 values varied widely for each sample; coefficients of variation for the 4 samples were 36.0, 51.0, 12.8, and 55.0%. Recovery of KNO_3 from the spiked sample averaged 105%. The analytical results, as a group, were unacceptable both statistically and for use in feeding recommendations. Three values reported could have resulted in feeding potentially toxic forage to livestock.

Nitrate poisoning has been suspected as a major cause of livestock losses for nearly a century (1), and forages are recognized as a common source of nitrate (2). The need for prompt, accurate nitrate determinations on forage samples is evident. The literature describes many methods including the ion-specific electrode method of Sweetsur and Wilson (3) and the AOAC official final action method (4) based on cadmium reduction. However, in our experience, wide variations in results occurred when several samples were split and analyzed for nitrate content by various laboratories. This finding prompted us to organize a laboratory performance study for forage nitrate analysis. The study was designed to allow evaluation of nitrate determination data from independent laboratories and to establish forage pools for our routine nitrate quality control.

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Table 1. Preliminary determination of KNO_3 in pool samples ($n = 3$)

Sample	KNO_3, %	CV, %
A	5.15	0.30
B	1.15	4.3
C[a]	3.49	2.3
D	0.34	5.9

[a]Sample D plus 3.1% KNO_3.

Table 2. Individual laboratory results for determination of KNO_3 (%) in pool samples

Lab.	Method	Sample A	Sample B	Sample C,[a]		Sample D
				% KNO_3	Spike rec., %	
1	SSE[b]	5.68	1.20	3.60	107	0.26
2	SSE	5.71	1.08	3.45	103	0.26
3	SSE	6.41	1.36	4.15	121	0.33
4	SSE	6.20	1.30	4.00	117	0.31
5	SSE	5.32	1.06	3.55	108	0.30
6	Cd[c]	5.9	1.6	2.8	86	0.14
7	SSE	7.44	2.81[d]	3.48	91	0.74[d]
8	Cd	5.05	1.50	4.36	131	0.23
9	Color.[e]	4.07	0.26	3.09	94	0.17
10	SSE	5.66	1.41	3.82	111	0.35
11	Cd	2.97	0.39	3.41	108	0.06
12	Cd	1.63	0.29	0.98[d]	31	0.07
13	Cd	5.36	0.91	3.23	99	0.15
15	SSE	3.83	0.72	2.89	90	0.10
17	Color.	0.94	0.10	0.45[d]	14	0.01
19	SSE	5.64	1.16	3.49	104	0.26
	Mean	4.86	0.96	3.52	105	0.20
	CV, %	36.0	51.0	12.8	12.0	55.0

[a]Sample D plus 3.1% KNO_3.
[b]Solid-state nitrate electrode method.
[c]Cadmium reduction method.
[d]Outliers excluded from calculations.
[e]Colorimetric method.

Table 3. Number of outlying values[a] for each KNO_3 pool sample for various ranges about mean

Range allowed, ± % from mean[b]	Sample A	Sample B	Sample C	Sample D
2	16	16	12	16
5	15	15	10	16
10	14	14	8	16
15	13	13	6	14
25	7	10	2	13

[a]16 values for each sample.
[b]Means as shown in Table 2.

Experimental

Reagent and Apparatus

(a) *Potassium nitrate.*—Certified ACS grade (Fisher Scientific Co.). Dried 2 h at 130°C before use.

(b) *Wiley mill.*—With 2 mm screen (Arthur H. Thomas Co.).

Sample Preparation

Samples of dried, ground forage including hay, straw, silage, corn-stalks, and grasses received by our laboratory for KNO_3 determination were saved over a period of 3–4 months. These samples were separated into 3 groups on the basis of KNO_3 content as determined by our solid-state electrode application of the extraction method of Baker and Smith (5). Samples in each of the 3 groups (<0.5% KNO_3, 0.5–2.5% KNO_3, and >2.5% KNO_3) were separately pooled in covered buckets and thoroughly mixed by rotation. Each pool was reground through the Wiley mill, returned to the bucket, and mixed again.

A portion (750 g) of the 0.5% pool was placed in another bucket and a solution of 23.25 g KNO_3 in approximately 2 L water was added. The resulting slurry was manually stirred for 10 min, then poured into pans, and dried overnight in a 60°C forced air oven in the same manner as the original forage. This pool was reground and mixed as before to yield a sample containing a uniform 3.1% KNO_3 spike. Each of the 4 pools was mixed again, split into 25–50 g portions, and given a letter designation. Three samples of each pool were selected at random and analyzed by solid-state electrode for KNO_3 content. The results (Table 1) indicate good homogeneity (coefficient of variation <6%) for all pools. Spike recovery was 101%.

Participants

Sixteen of 19 laboratories contacted agreed to participate. Each laboratory was assigned a number for identification to assure anonymity. We requested that each of the 4 samples be analyzed for KNO_3 content by the method of their choice. Replicate analyses were not required. Information was requested on the type of method used in the determination.

Results and Discussion

Table 2 shows individual laboratory results, including means and coefficients of variation (CV). Applying Dixon's test (6), we found 4 outliers which are excluded from subsequent calculations. The ranges of the remaining results for each sample are still wider than expected. Horwitz et al. (7) showed that collaborative studies on materials present in amounts of approximately 1% will generally result in a CV of 2–5%. Mason (8) stated that interlaboratory results within ±10–15% of the mean are acceptable. In our study, only the spiked sample C showed a CV of less than 15%, and this occurred only after the exclusion of 2 outliers. Table 3 illustrates the outlying results at various allowed ranges. It is important to keep in mind that all of the laboratories were aware that the samples were for interlaboratory study. Mason (8), in his evaluation of drug testing laboratories, documented the tendency of laboratories to provide better results on check samples than on "blind" samples. Thus, results on submitted field samples may have been worse than those reported for our pool samples. Three reported values of less than 1.0% KNO_3 were for forages that actually contained more than 3.0% KNO_3. If forages containing more than 1.6% KNO_3 are considered potentially toxic (2), these results could have allowed the feeding of toxic forage to livestock.

We feel that the variation in results reported in this study is unacceptable. It should be possible to achieve interlaboratory CV values of 15% or less on samples with this level of KNO_3 present. It is imperative to reduce interlaboratory variation in KNO_3 analyses to a reasonably low level (i.e., <15%

CV) if uniform guidelines are to be used in feeding recommendations.

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